THE ADDITIVE EFFECTS OF NAPHTHALENE, ACENAPHTHENE, BENZENE, AND 1-CHLORONAPHTHALENE ON MITOCHONDRIAL RESPIRATION

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CHAPTER I

INTRODUCTION

Testing of Multiple Environmental Contaminants

Aquatic toxicity testing has, in general, focused on results obtained by exposing organisms to single compounds. Only rather recently have a limited number of studies regarding mixture-toxicity testing appeared in the literature. Many of the United States Environmental Protection Agencies (EPA) water quality criteria, as mandated through the Clean Water Act (CWA) of 1981, the Resources Conservation and Recovery Act (RCRA) of 1980, and the Federal Water Pollution Control Act Amendment of 1982, have been established by testing individual compounds. However, the utility of these criteria, as discussed by Voyer et al. (1984) is limited by the fact that aquatic organisms are much more likely to encounter mixtures of toxicants rather than single compounds. Interactions between toxicants as well as interactions between toxicants and environmental parameters such as salinity, pH, etc., may also affect the toxicity of a mixture of compounds (Nelson, 1977).

Surface waters, ground waters, leachates, and effluents often contain hundreds of compounds. However, relatively

little is known regarding the effects of these multiple compounds on aquatic life or humans. Many of the recent studies regarding mixtures have focused on metals or low molecular weight, hydrophobic, aliphatic or aromatic hydrocarbons grouped into a class of compounds usually considered to cause a generalized narcosis (Deneer et al., 1988).

Further evaluations of aquatic pollution demand the need for the development of testing procedures which will provide the answers regarding mixture interactions as well as environmental factors which affect mixture-toxicity. This is necessary for the true assessment and understanding of environmental contamination.

For testing of mixtures to become practical, modeling procedures must be developed which will accurately describe the toxicity of a mixture based on the analysis of its individual compounds. As will be discussed in Chapter II, several types of interactions of compounds may take place and a few modeling procedures have been described.

In Vitro Toxicity Testing

The current methods of toxicity testing are slow and costly. Increasing concern regarding the pollution of the aquatic environment has led to an additional demand for quantifying contamination. To meet this demand, new, rapid, sensitive, inexpensive, and reliable testing procedures must be developed.

The trend is toward in vitro testing where the time

necessary to obtain results and the cost of operation are minimal. Assays utilizing a measurable parameter of metabolic energy production in an *in vitro* system seem to be a practical test since the generation of effective energy for the growth and maintenance of an organism is an absolute necessity for its survival. Nearly every biochemical reaction in a living organism relies on stored chemical energy generated during the normal processes of respiration and nutrient utilization. These processes and their implications for use as a testing tool are discussed in Chapter II.

Focus of Study

This work focuses on three important and current aspects of toxicity testing: mixture-toxicity testing, *in vitro* testing, and regression modeling of mixture data in order to develop a single predictive equation to describe the toxicity of multiple compounds. Each of the aspects of this study and the current status of the literature will be discussed in detail in Chapter II.

CHAPTER II

LITERATURE REVIEW

Mixtures of Compounds

General

As mentioned in Chapter I, the investigations into the toxicities of aquatic pollutants have dealt primarily with individual compounds. While it is desirable to know the effects of individual compounds, this information by itself does not describe a realistic situation. Compounds are not likely to be found alone in the aquatic environment but with hundreds of other compounds.

Three types of biochemical and physiological actions of mixtures were originally described by Bliss (1939) as additive, synergistic, or antagonistic. As Plackett and Hewlett (1948) point out, this is the result of the compounds having similar or dissimilar modes of action.

Additive Mixtures and Descriptive Models

Additive mixtures refer to those whose toxicity is simply the sum of the toxicities of the individual compounds present in the mixture. The joint toxicity of compounds which act similarly has been predicted using the model of

concentration addition (Plackett and Hewlett, 1952; Muska and Weber, 1977; Konemann, 1980). Compounds which are described as "narcotizing agents" include a large group of hydrocarbons and are generally thought to exert a relatively nonspecific effect via perturbation of biological membranes (Deneer et al., 1988; DeWolf et al., 1988; Hermans et al., 1984a; Broderius and Kahl, 1985). The toxicity of a mixture of these agents is predictable using the concentration addition model (CA). With this model the concentration of each compound is expressed in toxic units (TU). The TU's are calculated based on the fraction of the mean effective concentration (EC_{50}) of the compound present. The toxicity of the mixture is then calculated by summing the TU's for all the mixture components (Deneer et al., 1988; Sprague, 1970).

Several experiments using a variety of different aliphatic and aromatic compounds, metals, and etc., as well as different test organisms have been found to support the concentration addition model. Hermans et al. (1984b) analyzed mixtures of 14 aquatic pollutants with several chemical structures (PCP and malathion, for example) on the acute mortality and inhibition of reproduction in Daphnia magna. Data supported the hypothesis of concentration addition for the mortality study. However, compounds tested in the reproduction study caused an inhibition of reproduction slightly less than additive, though not appreciably. Mixtures of chlorophenols (Konemann, 1981), anilines (Her-

mans et al., 1984c), and the metals copper and cadmium (Westerhagan et al., 1979) were also shown to be additive.

In many cases the components of the mixtures in the CA testing were present at equitoxic levels or at concentrations above their "no observed effect level". Deneer et al. (1988) tested mixtures of 50 nonreactive organic compounds on D. magna such as benzene, acetone, chlorobenzenes, alcohols, diols, etc., at levels below the "no effect level". The finding opposed an earlier consensus made by the European Inland Fishery Advisory Committee (EIFAC) which stated that toxicants present at levels below 0.1 of their threshold lethal concentration made no contribution to the joint toxicity of the mixture. Deneer et al. (1988) showed, with compounds generally considered to act by a common, nonspecific action, no concentration below which these compounds did not contribute to the overall toxicity of the mixture. In other words, even at a very low concentration, below their no effect level, compounds which act similarly to other components in a mixture still contribute to the overall potency of the mixture. Mixtures of compounds which cause a generalized narcosis act in this manner (Broderius and Kahl, 1985; Deneer et al., 1988). The overall anesthetic potency of these mixtures is related to the sum of the constituents, no matter how low the concentration of individual components.

The anesthetic-like compounds were also tested by Broderius and Kahl (1985) using fathead minnows. Structure-

activity relationships (described in the next section) were used to estimate the anesthetic potency of 27 different compounds from seven different groups. The compounds were chosen based on their similarity from the structure-activity relationship. The results of these tests, like those using D. magna, also indicated that the compounds act additively. This additive effect was predictable using the model of concentration addition for compounds such as alcohols, ketones, ethers, tetrachloroethylene, chloropropane, etc.

Pesticides are often applied in mixtures. For example, chlordane, a previously used termiticide, contained other compounds such as heptachlor. Mixtures of pesticides have been tested by Matthiessen et al. (1988) on rainbow trout (Salmo gairdneri). Results were either additive, or slightly less than additive. There was no statistical evidence suggesting synergism.

Factors such as temperature, salinity, and pH have been shown by Brown (1968) to effect the toxicity of a mixture of compounds to rainbow trout. Increased temperature (above 13 degrees C) was shown to cause a decrease in the toxicity of ammonia, and HCN. Zinc, however was more toxic at higher temperatures. Many metals are bound to sediments or form metal-hydroxides at neutral to basic pH's in the aquatic environment. They are therefore not free to affect those organisms in the water column. As the pH becomes increasingly acidic the metals dissociate from the hydroxide complexes and enter into solution where organisms may be

exposed. Therefore, a mixture ordinarily expected to be additive may be synergistic or antagonistic based on the physico-chemical conditions of the surrounding solution.

QSAR Models for Prediction of Concentration Addition

The use of Quantitative Structure-Activity Relationship (QSAR) models has been relatively popular for describing the effects of mixtures of compounds with anesthetic potency (Hermans et al., 1984a; DeWolf et al., 1988; Foster et al., 1984). QSAR's allow the prediction of EC_{50}'s based on a molecular characteristic such as hydrophobicity (which may be determined from solvent/water partitioning experiments). The toxicity of mixtures have been estimated by expressing the fraction (based on concentration) of each EC_{50} present in the mixture. These estimates may actually be tested for verification of accurate prediction. IC_{50}'s (the concentration required to cause 50 % immobilization of the test organism) have also been predicted using QSAR and hydrophobicity (Foster et al., 1984).

QSAR models were used by DeWolf et al. (1988) to calculate the EC_{50} of 10 organic compounds. A comparison was made between predicted (QSAR) mixture toxicity and actual results from *D. magna* bioassays. The toxicity of the mixture was additive and the QSAR's quite predictive.

Hermans (1984a) also used QSAR's and hydrophobicity to predict the toxicity of mixtures of 50 compounds to *D.*

magna. No observable deviation between the bioassay results and the predicted concentration addition given by the QSAR was reported.

Synergistic Mixtures

Synergistic effects may also occur with mixtures of compounds. If compounds act synergistically then the effect of the mixture is greater than would be expected based solely on the sum of the toxicities of the mixture components. A classical example of this is the mixture of pipernyl butoxide with the pyrethroid insecticides commonly used in flea shampoos. The pyrethroids are rapidly detoxified via the hepatic mixed function oxidase (MFO) enzyme system, and, are thus, relatively nontoxic. When pipernyl butoxide (which alone is also relatively nontoxic) is added to the application the result is an extremely toxic mixture. This is due to the inhibition of MFO's by pipernyl butoxide, allowing the pyrethroids to exert their effects.

Metals such as copper and silver have been shown by Coglianese and Martin (1981) to have an effect which is greater than additive.

Synergistic Models

As one might imagine attempts to model synergistic effects are quite involved and complex, and thus, few have been noted in the literature. Factorial design (Box et al., 1978; Zolsnay et al., 1987) has been recently presented as a

possible modeling scheme for estimating synergistic effects with the ultimate goal of a predictive equation. All variables (compounds) can be considered simultaneously and not only main effects but the presence or absence of synergism may also be detected. If the mixture is synergistic or antagonistic appropriate interaction terms may be incorporated into the predictive equation. This, however, is limited to extremely simple mixtures as factorial design requires an enormous amount of data per variable. An experiment with 3 compounds at 6 concentrations would require 3^6 data points.

Antagonistic Mixtures

Antagonistic mixtures are those which are less toxic than would be expected from an additive mixture. This could occur if one chemical competitively inhibits another, or induces the detoxification of that compound.

Some pesticide mixtures (Matthiessen et al., 1988) have been shown to be antagonistic having a toxicity of 50 percent of that based on additivity. Mixtures of metals such as copper and mercury (Moulder, 1980) or lead and mercury (Gray, 1974) have also been observed to be antagonistic (sometimes called negative synergism).

Multiple Regression as a Model

Voyer and Heltshe (1984) suggested the use of multiple regression in modeling the effects of mixtures. However, no

literature citations were observed which dealt with the application of regression to actual mixture testing. This study, as described in Chapter III, uses multiple regression to develop predictive equations relating to mixture-toxicity. Regression allows the mathematical description of of each mixture components individual effect, and also the incorporation of these individual effects into a larger "mixture equation". If the interaction is other than additive, interaction terms may also be determined statistically and incorporated into the regression model.

In Vitro Toxicity Testing

General

With the regard to the future demand for increased toxicity testing, new and rapid in vitro tests for screening compounds, mixtures, and complex effluents will be an absolute necessity (Oesch et al., 1988). The expanding or commercial use of in vitro tests depends on their development as accurate models of toxicity in vivo (Flint, 1988), without which they lack real value. With increasing pressure to get away from the use of animal testing, in vitro studies appear to be an attractive alternative.

Current methods of quantifying aquatic toxicity rely chiefly on the use of fish or aquatic invertebrates. These tests range in cost from $500 to $1500 and require from 24-96 hours to obtain acute toxicity ($LC_{50's}$) data.

Therefore, these tests lack the practicality to form the basis of an extensive water quality testing regimen (Blondin et al., 1987).

Current Approaches

In vitro testing methods utilizing bacterial responses are leading the way into this new type of testing. Examples of this are the 5-minute Beckman Microtox^{tm} test (Blondin et al., 1987; Chang et al., 1981) and respiration and growth assays using Pseudomonas flourenscens (Trevors et al., 1981; Trevors, 1982). The Beckman Microtox^{tm} test based on light emissions from Photobacterium phosphoreum was judged to be the most sensitive and fastest (Dutka and Kwan, 1981). Blondin et al. (1985), however, noted that the Microtox^{tm} test compromised speed for sensitivity and reproducibility, and that the test correlated poorly (r^2=29%) with the fat-head minnow bioassay (EPA standard test).

In Vitro Mitochondrial Assays

Blondin et al. (1987) recently suggested the use of mammalian mitochondria as in vitro monitors of aquatic samples and demonstrated the use of submitochondrial particles and energy-coupled reverse electron transport as a bioassay. With this assay the reduction of NAD^+ is monitored spectrophotometrically at a wavelength of 340 nm. Compounds which affect electron flow would decrease the reduction of NAD^+ and would be assessed as having a toxic

effect since they block mitochondrial electron transport. The correlation of the mitochondrial assay (Blondin et al., 1987) to the fathead minnow bioassay was much higher (r^2=82 %) than was the Microtox^{tm} test (r^2=29 %).

Proposed in this study is the measurement of oxygen consumption *in vitro* in intact, isolated mitochondria. This is a truly vital biochemical process, as mentioned below, and is fairly easy to perform. The oxygen consumption assay also encompasses a greater number of vital enzyme systems which may be affected by environmental samples than the reverse transport assay.

Mitochondrial Function and Energy Production

As mentioned in Chapter I, an *in vitro* system which measures a physiological or biochemical parameter vital to overall organismal well-being is more likely to be correlable to whole-organsism toxicity. Harmon and Sanborn (1982) demonstrated the parallel between the effects of naphthalene on mitochondrial function, cell cultures, *D. magna*, and fathead minnows.

The mitochondrion is the cellular site for the utilization of molecular oxygen and metabolic energy production in the eukaryotic organism. Energy production begins in the cytoplasm of the cell where the six-carbon sugar glucose is converted via the glycolytic pathway into two molecules of pyruvate. Two molecules (net) of the energy rich complex

adenosine triphosphate (ATP) are formed during glycolysis, but it is in the mitochondrion where oxidative phosphorylation takes place that the bulk of the necessary ATP formation occurs. Fatty acid breakdown also occurs in the mitochondria; the acetyl CoA generated is used elsewhere in the cell for such processes as acetylcholine (an essential neurotransmitter) production (Harmon, 1988b). Many precursor molecules are offshoots of mitochondrial metabolic pathways. Mitochondria also regulate intracellular calcium concentrations. Thus, alterations in normal mitochondrial function may have diverse effects within the organism.

In the mitochondrion the oxidation of energy-rich reduced molecules and the reduction of molecular oxygen to water is coupled to the phosphorylation of adenosine diphosphate (ADP) to form ATP. The high energy ATP produced is used as an energy storage and currency molecule and enables an organism to do chemical, physical, and osmotic work (Tzagoloff, 1983). This production of ATP is known as oxidative phosphorylation and in this respiratory process (Mitchell's chemiosmotic hypothesis, 1974) a membrane potential an a pH gradient are established across the inner mitochondrial membrane. This pH gradient and membrane potential are collectively called the proton motive force (PMF) which generates energy for ion transport and ATP synthesis. The major component of the PMF is the pH gradient (H^+ outside).

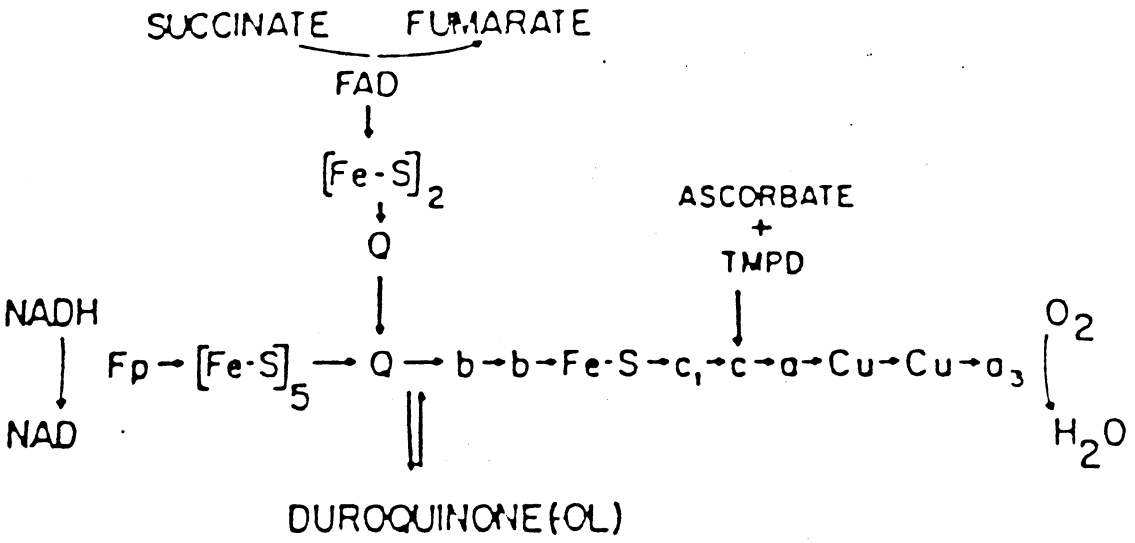
Many different substrates are utilized within the

mitochondrion. NADH, for example is formed during the oxidation of pyruvate via the Krebs' cycle and succinate which is also oxidized during the Krebs' cycle provide electron sources to the mitochondrial respiratory chain as described below. Many other artificial substrates have been found to be oxidizable by the respiratory chain (duroquinol, for example) and may be used to probe various portions of the chain. Mitochondria from different tissues may also vary in their substrate utilization. Brain and heart mitochondria are highly glycolytic and utilize pyruvate, while liver mitochondria are less discriminating.

Mitochondrial Respiration

The mitochondrial respiratory chain is diagrammed in Figure 1 and consists of four large enzyme complexes designated I-IV. The four complexes which are located in the mitochondrial inner membrane carry out a stepwise catalytic transfer of electrons which results in the harnessing of energy (in the form of the terminal pyrophosphate bond of ATP). If the electron transfer process was to occur in one large step much of the energy produced would be lost as heat. In addition to the respiratory complexes, two mobile electron shuttles transfer electrons between the complexes (Tzagoloff, 1983). Coenzyme Q, a lipoidal quinone, acts as a shuttle between complexes I-III and complexes II-III. Cytochrome c, a heme-protein, acts as a shuttle between complexes III-IV. Both of these shuttles may pick up elec-

Figure 1. Mitochondrial electron transport chain. Abbreviations: Fe-S, iron-sulphur proteins; Fp, flavoprotein; Q, ubiquinone; TMPD, N, N, N', N -tetramethylphenylenediamine.


SUCCINATE
FUMARATE
FAD
[Fe-S]2
Q
ASCORBATE
+
TMPD
NADH
NAD
Fp → [Fe-S]5 → Q → b → b → Fe-S → c1 → c → a → Cu → Cu → a3
O2
H2O
DUROQUINONE(OL)

trons by becoming reduced and then transfer the electrons as they are oxidized. Each of the respiratory complexes and their components are discussed below.

Complex I is known as NADH-ubiquinone reductase and contains NADH dehydrogenase as well as a flavoprotein and several iron sulfur (Fe-S) centers which transfer electrons to ubiquinone (coenzyme Q). Complex I is composed of some 10 different protein subunits some of which are associated with an NADH dehydrogenase and nonheme iron protein (Hatefi, 1985). NADH acts as the substrate giving up electrons as it is oxidized to NAD^+ via the NADH dehydrogenase and its associated FMN prosthetic group and iron-sulfur center. Electrons pass through at least three more iron-sulfur centers (denoted FeS_2, FeS_3, and FeS_4) to coenzyme Q.

Complex II is the succinate-ubiquinone reductase complex. Complex II is considered a respiratory complex, however it is also responsible for the oxidation of succinate to fumarate in the Krebs' cycle. The complex contains 7-8 nonidentical subunits ranging in molecular weight from 12,000 to 70,000. These subunits include a flavin, nonheme iron, and cytochrome b_{558}. According to Tzagoloff (1983) the stoichiometry of the FAD and 3 nonheme Fe-S centers is 1:1:1:1 and the function of the b-type cytochrome is unknown. The succinate dehydrogenase contains as its prosthetic group FAD (flavin adenine dinucleotide) which accepts electrons as succinate is oxidized to fumarate. The electrons are transferred via the Fe-S centers to coenzyme

Q. Coenzyme Q transfers electrons to Complex III.

Complex III is known as both the coenzyme-QH_2-cytochrome c reductase complex or the b-c_1 complex. In Complex III the reduction of cytochrome c by coenzyme QH_2 occurs. This complex contains as its essential redox components two b-type cytochromes denoted by their alpha-band absorbance maxima (b_{562} and b_{566}), an Fe-S cluster known as the Reiske center, and cytochrome c_1 in a 2:2:1 stoichiometry (Houslay and Stanley, 1982). At least eight subunits have been identified with the function of those not mentioned already still undetermined. Reduced coenzyme Q is oxidized to semiquinone by cytochrome b_{562} and then further oxidized by cytochrome c_1. One of the electrons removed from the reduced Q is cycled between cytochromes b_{562} and b_{566} and therefore there is a net transfer of only one electron per cycle with the transport of two protons across the membrane. This model, proposed by Mitchell (1975a and 1975b) is known as the Q-cycle. Cytochrome c_1 passes the other electron to cytochrome c which shuttles the electrons to Complex IV, the cytochrome oxidase complex.

Cytochrome oxidase is a membranous, multisubunit complex with a molecular weight of approximately 200,000 daltons and is composed of at least seven non-identical subunits. The essential redox components include two a-type cytochromes (a & a_3) and two copper atoms. Electrons from cytochrome c are transferred to cytochrome a located on the cytoplasmic face of the membrane via the copper atoms to

cytochrome a_3. Cytochrome oxidase is the terminal oxidase in the mitochondrial electron transfer chain and contains in its active state about 20 % phospholipid (Yu et al., 1985). The lipid requirement for cytochrome oxidase function will be discussed below. Complex IV is the site of cytochrome c oxidation and the utilization of oxygen in organisms.

Electrons pass down the respiratory chain via an increased affinity of the redox components of the chain for the electrons (due to an increasing electromotive midpotential). The midpotential, based on the hydrogen couple set at O mV, is used to measure the relative potential for a compound to accept or donate electrons. Compounds with a negative midpotential (less than hydrogen) tend to donate electrons (act as reductants) while compounds with increasingly positive midpotentials become stronger oxidants and like to accept electrons. The sequence of components of the respiratory chain are arranged in order of increasingly positive midpotential. Therefore, as substrates such as NADH, which has a midpotential of approximately -430 mv, donate electrons to the respiratory chain; these electrons are transferred to the ultimate electron acceptor molecular oxygen, which has a midpotential of +800 mv (Alberts, et al., 1983).

There are three places in the respiratory chain where decreases in redox potential are large enough to drive ATP synthesis: complex I, III, and IV. A potential decrease can be translated into a free energy release (ΔG^{o}) which is

related to the equation $\Delta G^{o} = -nF \Delta E_{o}$, where F is the Faraday constant (23.062 kcal), n is the number of electrons involved, and ΔE_{o}, is the magnitude of difference between redox components (Tzagoloff, 1983). The energy released by these decreases is harnessed by the enzyme complexes to pump protons across the inner membrane, generating the PMF (Alberts et al., 1983). ATP synthesis (from ADP and inorganic phosphate) occurs as these protons reenter the membrane via the F_0F_1-ATP synthetase. For ATP formation to occur (ie. the addition of the final pyrophosphate ester to ADP), the standard free energy change is estimated as 7.3 kcal. NADH oxidation provides a release of 52.6 kcal and therefore, should produce enough energy to synthesize (52.6/7.3) 7.2 moles of ATP per mole of NADH (Tzagoloff, 1983). However, the oxidation of one mole NADH results in only 3 moles of ATP produced and thus this process is only 40 % efficient. ATP formation, as mentioned above, takes place in complexes I, III, and IV where redox potential decreases of 220 mv or more and corresponding free energy changes sufficient to synthesize ATP may occur. In Complex II, the succinate-Q reductase complex, the redox potential decrease is 65 mv (Whittaker and Danks, 1978). This corresponds to a ΔG^{o} of only -2.9 kcal and thus there is insufficient energy to drive ATP production in this respiratory complex.

The importance of electron transport in producing the free energy for the formation of ATP is demonstrated above.

Compounds which alter the passage of electrons in the mitochondria or cause changes in the electrochemical gradient established through respiration will affect oxygen consumption and ATP generation and are of obvious concern.

Mitochondrial Alterations

Compounds which alter mitochondrial respiration and energy production are of three main types: inhibitors, uncouplers, and detergents. Inhibitors act by blocking an oxidation-reduction step in the electron transfer chain and decrease oxygen consumption. This block occurs via a specific interaction with a component of the chain. Oxygen consumption and ATP (oxidative phosphorylation) formation have a fixed stoichiometry (Tzagoloff, 1983). The efficiency of phosphorylation is expressed by P/O ratios and for NADH as a substrate this ratio is three (three ATP produced per molecule of NADH). When oxygen consumption is inhibited the phosphorylation thus decreases proportionately and the P/O ratios do not change. Rotenone is a classical inhibitor. It acts in complex I before coenzyme Q but after NADH and FMN. Antimycin A, a microorganism derived compound is a potent inhibitor of complex III and inhibits respiration at 10^{-8} M. Cytochrome b will reduce in the presence of antimycin A, but c_1, c, and complex IV remain oxidized in the presence of NADH and O_2. Naphthalene also acts as an inhibitor and was shown by Harmon and Sanborn (1982) to block the the reduction of coenzyme Q.

Uncouplers generally act as lipophilic weak acids and dissipate the pH gradient established during respiration. They effectively "uncouple" respiration from oxidative phosphorylation. Respiration occurs at a normal or accelerated rate, but no energy exists to produce ATP from ADP and inorganic phosphate. Therefore, the P/O ratios drop in the presence of an uncoupler. Dinitrophenol, pentachlorophenol, and carbonyl cyanide m-chlorophenylhydrazone (CCCP) are classic uncouplers. The uncoupler CCCP is so potent that it halts phosphorylation at 10^{-7} M.

The difference between uncouplers and inhibitors is that inhibitors affect electron transport while oxidative phosphorylation still occurs at a slower rate due to the fixed stoichiometry between oxygen consumption and phosphorylation. Uncouplers do not affect electron transport or oxygen consumption, but they do prevent phosphorylation by dissipating the energy source required for bond formation.

Detergents (amphiphiles) interact with biological membranes and proteins by solubilizing into their hydrophobic interior. This may affect the mitochondrial inner membrane or its associated proteins, by disrupting their function and the ability to participate in electron transfer. Many proteins are intimately associated with certain lipid molecules and this association is vital for the function of the protein. If a detergent perturbs these "annular" lipids the function of the protein may be affected.

Cytochrome c oxidase (Complex IV) of the mitochondrial

electron transfer chain requires annular or boundary lipids. If the oxidase is isolated and lipid-depleted it is enzymatically stable and has spectral properties similar to that in the intact mitochondria, but lacks activity (Yu et al., 1985). Differential scanning calorimetry (Yu et al., 1985) show the oxidase has specific boundary phospholipids associated with the complex. Chuang et al., (1970) was able to show restoration of lipid-depleted oxidase activity in the presence of amphiphilic detergents which essentially act as phospholipids. The structural similarity of many of the detergents with phospholipids enables them to effectively partition into the membrane bilayer. Since proteins have a hydrophobic core detergents may also partition into the protein and disrupt its tertiary and quarternary conformation. This obviously may affect function.

In this study only compounds acting as inhibitors of electron transport were examined. Oxygen consumption assays (as described in Chapter III) were performed to measure the effects of these compounds on electron transport.

Sites of Action in the Mitochondrial Respiratory Chain

Once a chemical is determined to be a respiratory inhibitor a site of action within the electron transport chain is desirable. This is accomplished by utilizing different substrates or oxidants (artificial or natural) which transfer or remove electrons in the respiratory chain

at different sites. This effectively breaks down the respiratory chain into several partial reactions so the site of action can be determined. Using NADH as substrate and measuring oxygen consumption enables the analysis of complexes I, III, and IV. Succinate as substrate allows the analysis of complexes II, III, and IV. Ascorbate and N, N, N', N'-tetramethyl-p-phenylenediamine (TMPD) as reductant allow analysis of only the terminal oxidase portion of the respiratory chain (complex IV). Each of the complexes may be further broken down. Using coenzyme Q with dithiothreitol as the electron source encompasses the chain from coenzyme Q to oxygen. Duroquinol (Figure 1) with dithiothreitol as reductant analyzes that portion of the respiratory chain on the oxygen side of coenzyme Q (Hare and Crane, 1971). Reaction sequences that encompass several complexes are generally analyzed initially and the partial reactions are progressively used to focus on the site of action. Many of these partial reactions may be measured polarographically while others, not based on oxygen consumption (NADH-ubiquinone reductase, for example), may be examined spectrophotometrically.

Interactions Between Hydrophobic Contaminants and Cellular or Subcellular Material

The group of aromatic hydrocarbons under examination in this study (see below) are representative of hydrophobic

molecules which may basically act at either membranes and proteins, both of which have hydrophobic portions.

Membranes are composed of amphiphilic phospholipids arranged in a bilayer with their charged hydrophilic surfaces in contact with the polar aqueous medium surrounding them. They also contain a hydrophobic portion which, due to the high dielectric constant of water and the energetically unfavorable interaction of the apolar hydrocarbon tails of phospholipids with the aqueous medium, remains buried in the hydrophobic core of the bilayer. The low energy state achieved by aggregating the non-polar regions of the lipids helps to maintain a stable configuration and the exclusion of water acts to provide the hydrophobic force holding the membrane together. It is this hydrophobic core which provides the barrier to transport of polar compounds.

Intrinsic and extrinsic membrane proteins or globular proteins free in the cytosol contain many hydrophobic and hydrophilic amino acid residues. The three dimensional structure of the protein is such that the hydrophobic regions are buried within the interior of the molecule. Hydrophobic molecules which may affect the structure/function of membrane bound or soluble proteins may contribute to deleterious effects on the entire organism. Mitochondrial proteins, if altered, may affect energy transformation which may produce toxic effects in the organism. As mentioned above many proteins are also dependent on their interaction with surrounding phospholipids. Alteration in this lipid-

protein interaction may also affect protein function.

Many organic pollutants are hydrophobic and are readily soluble in the lipid interior of membranes and the hydrophobic core of proteins. Here they may act to perturb the molecular structure and alter the properties of the membrane, such as increasing or decreasing its fluidity (Nielson et al., 1979; Packham et al., 1981). Binding of hydrophobic molecules to particular sites on membrane surfaces may affect cell function by affecting membrane bound enzyme systems or membrane receptor proteins. Aromatic hydrocarbons have been shown by Roubal and Collier (1975), via spin labeling, to disrupt cellular membranes by selectively partitioning into the membrane. Roubal and Collier (1975) have also shown that those hydrophobic molecules which partition into, but fail to penetrate the membrane are the most toxic. These compounds may act by a specific interaction with a membrane bound component of a cellular enzyme system, a receptor for a second messenger system, etc.

As mentioned, a great deal of research has been performed on the group of hydrophobic compounds generally thought to produce a narcotic-like effect. The assumption with the <u>in vivo</u> testing methods used to look at the affect of these "narcotizing agents" is that they act similarly by perturbing the cell membrane (DeWolf et al., 1988; Hermans et al., 1984a). Using the mitochondrial assays described above the effects of these compounds on several enzyme

systems can be measured to see if the effect is generalized or specific. Further spectrophotometric analysis of mitochondrial components may also establish specificity.

Compounds Selected for Mixture Testing

General

Many studies have been performed on the deleterious effect of compounds which are found in the aquatic environment. These include metals, pesticides, and a variety of aliphatic and aromatic hydrocarbons. Many of the organic contaminants are the relatively low molecular weight fractions produced during petroleum processing whose toxicity has been directly correlated to their hydrophobicity (Konemann 1981a; Veith et al., 1983; Hermans et al., 1984a). These compounds are thought to act through a common mode of action referred to as narcosis (Deneer et al., 1988; DeWolf et al., 1988; Hermans et al., 1984a).

The relatively low molecular weight aromatic hydrocarbon benzene is common in petroleum wastes as are several slightly water soluble polynuclear aromatic hydrocarbons (PAH's). Produced through the pyrolysis of fossil fuels, PAH's are a class of compounds generally considered hazardous, toxic, and some are potentially carcinogenic (Lofroth, 1985). The aromatic portion of these wastes has been shown by Anderson et al. (1974) to be the most toxic of all the oil fractions, and well known is the fact that many hydro-

carbons induce physiological and biochemical changes (Roubal et al., 1977). The U. S. Environmental Protection Agency (EPA) lists 16 PAH as priority pollutants which may potentially harm the environment (Michelcic et al., 1988a, Dominguez, 1977; Bastian and Toetz, 1982). Since these chemicals are introduced by a variety of anthropogenic sources into the aquatic environment in complex mixtures, their interactions as well as biochemical effects need understanding (Michelcic et al., 1988b).

Chlorinated polynuclear aromatics are also found in environmental pollution from petroleum sources (Eklund et al., 1983). These compounds would be expected to occur in mixtures with other non-halogenated polynuclear aromatics.

This study examines the effects of the priority pollutants benzene, naphthalene, acenaphthene, and 1-chloronaphthalene on mitochondrial respiration individually and in mixtures. Naphthalene and benzene were chosen because of their ubiquitous nature and numerous literature citations. While acenaphthene and 1-chloronaphthalene have not been as extensively researched as naphthalene and benzene, they also require monitoring under EPA regulations and would be expected to occur in the same wastes as naphthalene and benzene. These molecules are related structurally as simple substituted or nonsubstituted aromatics. Their study also provides information regarding the effect of small structural changes (such as mono-halogenation) on toxicity. All four of the compounds belong to the class of compounds

sometimes referred to as general narcotizing agents. The literature related to the narcotizing effect of these types of compounds (DeWolf et al. 1988; Broderius and Kahl, 1985) makes the assumptions that these chemicals exert their effect via a nonspecific membrane perturbation and that this "common", nonspecific mode of action is the basis for their additive effect. The general, reversible, membrane perturbation has only been cited as the probable biochemical or physiological mechanism of action for these compounds (Klaassen et al. 1986), and no mention has been made regarding any specific affect on a membrane component(s). This work will establish the effect of these compounds on mitochondrial respiration and demonstrate a very specific interaction of the compounds individually which will explain their effects in mixtures.

Naphthalene

Naphthalene is considered to be a common and ubiquitous pollutant found in oil spills, coal tar, drinking waters, refined oil products, and industrial effluents (Harmon and Sanborn 1982; Struble and Harmon, 1983, Darville et al., 1983; and DeGraeve et al., 1982). Naphthalene is common in the water soluble fraction of petroleum waste with an approximate water solubility of 30 parts per million (ppm), a log P of 3.30, and a molecular weight of 128.18. According to Anderson et al. (1974) naphthalene and alkylated naphthalenes likely comprise the majority of the toxic water

soluble fraction of petroleum.

Naphthalene has been widely researched and is known to be lethal to freshwater organisms with an LC_{50} of 17.0 ppm to D. magna (LeBlanc, 1980) and an LC_{50} of 8.00 ppm to the fathead minnow (U. S. EPA, 1980b). Darville et al. (1983) reported that naphthalene disrupted ionic regulation and inhibited the Na^+-K^+ ATPase in Chironomus attenuatus. Hefler and Blankemeyer (1989) using isolated frog skin observed a dose related increase in Na^+ transport at doses above 1 mg naphthalene. Naphthalene was shown to affect the entry side of the frog skin which contains amiloride-sensitive sodium transport channels. No effect was observed on the serosal side of the membrane which contains the Na^+-K^+ ATPase. A possible second messenger effect is being hypothesized (Hefler and Blankemeyer, 1989).

Harmon and Sanborn (1982) examined the cellular and subcellular effects of naphthalene using intact cultured cells and isolated intact mitochondria. Naphthalene was shown to inhibit mitochondrial respiration through a specific interaction with coenzyme Q of the mitochondrial electron transfer chain and had an EC_{50} of approximately 10 ppm (78 uM). Oxygen consumption in intact cells was also inhibited parallel to the mitochondrial toxicity. Mitochondrial respiratory inhibition, as described above, was suggested as one likely mechanism for the observed effect at the cellular level (Harmon and Sanborn, 1982).

Crider et al. (1982) demonstrated a reduction in oxygen

consumption and hemoglobin content in D. magna in the presence of naphthalene. The decreased hemoglobin synthesis in the presence of naphthalene was speculated to be a result of decreased need due to reduced oxygen consumption.

Struble and Harmon (1983) described the molecular basis for the inhibition of coenzyme Q of the mitochondrial respiratory chain by naphthalene. The spectrum of the coenzyme was altered in the presence of naphthalene which demonstrates a rather specific interaction with coenzyme Q itself. The alteration of coenzyme may be responsible for the respiratory inhibition observed by Harmon and Sanborn (1982) and may affect the ability of the coenzyme itself to carry electrons or to bind Q-binding protein (Yu et al., 1977).

Cytochrome oxidase kinetics are altered by naphthalene (Harmon, 1988). Both the affinity of the enzyme (Km) for its electron donor, cytochrome c, and the maximum velocity (Vmax) of the reaction increase two-fold. However, since $v/Vmax = [S]/Km + [S]$ at a given substrate concentration [S], no net effect on the overall activity of the reaction is observed (Harmon, 1988).

Acenaphthene

Acenaphthene (1,2-dihydro-acenaphthalene or 1,8-ethylene-naphthalene) was chosen for this study because of its similarity to naphthalene in structure, its listing as a priority pollutant and because relatively little research

has been done regarding possible physiological effects of the compound (Cairns et al., 1982; Hall et al., 1986; Michelcic et al., 1988b). It is a fairly hydrophobic aromatic (log P = 3.5-4.0), has a molecular weight of 154.21, and a water solubility of 3.93 ppm. It has been used as a dye intermediate, a pesticide, in plastic manufacturing (U. S. EPA, 1980a), and has been produced during the coking of coal (Andreikova and Kogan, 1977). Acenaphthene has been found in urban air (Cleary, 1962), cigarette smoke (Severson et al., 1976), and well water (Burnham et al., 1972).

Acenaphthene has been shown by Cairns and Nebeker (1982) to affect the fathead minnow (Pimephales promelas) in flow-through toxicity tests with a 96 hour LC_{50} of 0.6 ppm. D. magna are less sensitive with a 48 hour LC_{50} of 41 ppm (Leblanc, 1980). Acenaphthene vapors have been shown by Imshenetskii et al. (1985) to increase the frequency of formation of morphological mutants in cultured cells of Candida scottii.

Benzene

Benzene is a low molecular weight (78.11), hydrophobic (log P = 2.13) hydrocarbon composed of a single aromatic ring. It is found in a variety of fossil fuels and effluents (DeGraeve et al., 1982) as well as throughout the petroleum industry. As the move to reduce tetraethyl lead from gasoline has increased, more and more small aromatics including benzene and toluene have been added which in-

creases the possibility of human exposure (Kalf, 1987).

Benzene has been shown to produce a variety of toxic manifestations. Increased incidence of myelogenous leukemia and some of its variants have been correlated to benzene exposure (Kalf, 1987). Benzene has also been shown to inhibit RNA synthesis and bind DNA in mitochondria (Kalf, 1982). Andrews et al. (1977) have shown that benzene causes decreased erythrocyte production in bone marrow.

Benzene, because of its ubiquitous nature and the fact that it has been shown to inhibit oxygen consumption in Mytilus californianus (Sabourin and Tullis, 1981), was chosen as a test compound. It has an LC_{50} (24 hr.) of approximately 250-300 ppm to D. magna (LeBlanc, 1980).

1-Chloronaphthalene

Relatively few literature citations describing the biochemical and physiological effects of 1-chloronaphthalene were found in this literature search. Chlorinated polynuclear aromatic hydrocarbons such as 1-chloronaphthalene are present in some combustion emissions since chlorine compounds are a natural constituent of many fuels (LoFroth et al., 1985). 1-Chloronaphthalene has been shown not to exhibit mutagenicity (LoFroth et al., 1985), and has an LC_{50} of 3.6-10.0 ppm to D. magna (LeBlanc, 1980). 1-Chloronaphthalene was chosen in this study to see the effects of a mono-chlorinated aromatic in mixture with other non-chlorinated aromatics.

CHAPTER III

MATERIALS AND METHODS

Mitochondrial Preparation

Intact bovine heart mitochondria were prepared as described by Harmon and Crane (1973). Fresh bovine hearts were obtained from a large slaughterhouse and put on ice. Cardiac muscle tissue was initially prepared by removing all excess fat, connective tissue, and inner (endocardium) and outer (visceral paracardium) covering layers. Small pieces of the prepared tissue were placed into a chilled commercial meat grinder and ground.

Ground tissue was placed in grind medium consisting of 0.35 M sucrose, 0.01 M dibasic sodium phosphate, 1 mM succinate, and 7.5 mM magnesium chloride. Dibasic sodium phosphate was added to adjust the pH to between 7.0 and 7.2. The mixture was then homogenized for 45 seconds with a Polytron^{R} scissors grinder to disrupt the cells. The homogenate was centrifuged at 1500 rpm; the supernatant contains the cellular constituents and the pellet contains mostly broken cell membranes. The supernatant was strained through a layer of cheesecloth and the pellet discarded.

The strained supernatant was centrifuged at 15,000 rpm in Sorvall RC-3B supercentrifuge. The pellet obtained was

homogenized using a teflon-glass homogenizer and resuspended in grind medium and centrifuged at 15,000 rpm. The pellets obtained contain the mitochondrial fraction and may be further purified by additional washings. Mitochondria were washed in KCl containing medium to yield cytochrome c depleted mitochondria. These isolated mitochondria are capable of both ADP-phosphorylation and proton translocation. Aliquots of 1 ml volume were stored in a -40 degree C freezer until needed.

Protein concentration of the prepared mitochondria was determined by the double Biuret assay (Yonetani, 1961) using bovine serum albumin (BSA) as a standard.

Oxygen Consumption Assays

NADH oxidase, succinate oxidase, duroquinol oxidase, and cytochrome c oxidase [using ascorbate and N,N,N',N -tetramethyl-p-phenylenediamine (TMPD) as electron donors] activities were assayed as previously described by Harmon and Crane (1976) by measuring oxygen consumption at 25 degrees C using a glass water-jacketed chamber fitted with a Clark oxygen electrode. The assay medium consisted of 77 mM sodium phosphate and 77 uM EDTA (pH 7.4), 100 ug intact mitochondria, 8.3 uM cytochrome c Type III, 2.6×10^{-7} M carbonyl cyanide-m-chlorophenylhydrazone (CCCP) to insure uncoupling, and water to fill to 1.95 ml volume. Absolute ethanol was also included in the assay medium in an amount which varied according to the amount of test compound added

(compounds were dissolved in ethanol) to yield a final volume of ethanol/ethanol-compound of 50 ul.

The substrates used in the assays were NADH at concentration of 0.5 mM for the NADH oxidase assays, 25.6 mM succinate for succinate oxidase, 0.6 mM ascorbate and 26 uM TMPD for cytochrome c oxidase, 77.9 mM duroquinol for the antimycin-sensitive duroquinol oxidase, and 53.5 uM ubiquinone-50 for antimycin-sensitive quinol oxidase. Dithiothreitol (Cleland's Reagent) was used as an electron source in the quinol and duroquinol oxidase assays at the concentration of 1.25 mM and 3.33 mM respectively.

Spectrophotometric Assays

Enzymatic reduction of ubiquinone (coenzyme Q) was assayed spectrophotometrically at 25 degrees C by following the disappearance of NADH at 340 nm by the method of Harmon and Crane (1976) using either a Gilford Model 252 single beam spectrophotometer or a Varian DMS-100S double beam spectrophotometer. The assay medium consisted of 100 mM sodium phosphate and 1 uM EDTA as buffer, 50 ug intact mitochondria, 9.35 nM antimycin A, 25 ug ubiquinone-50, 0.13 mM sodium cyanide, 0.35 mM NADH, 25 ul absolute ethanol/test compound dissolved in ethanol, and water to fill to a volume of 1 ml.

The ultraviolet spectrum of Ubiquinone-50 was analyzed using a double beam scanning Varian DMS-100S spectrophotometer equipped with a DS-15 data station. The spectrum of the

coenzyme in the presence or absence of perturbant molecules was recorded. The assay medium consisted of 1.93 uM ubiquinone-50, and either 50 ppm naphthalene (Struble and Harmon, 1983), 35 ppm acenaphthene, 30 ppm 1-chloronaphthalene, or 1545 ppm benzene, with absolute ethanol added to reach a 3.0 ml volume.

Preparation of Test Compounds

Naphthalene, acenaphthene, benzene, and 1-chloronaphthalene were dissolved in absolute ethanol and prepared fresh daily in 3000 ppm and 300 ppm stock solutions. These solutions were added to the assay medium to yield the desired final concentrations of the compounds. Inhibition of respiratory activity in the presence of these compounds was recorded between values of 0 and 95 percent or up to the approximate water solubility of the compound.

Regression Analysis

Data regarding the individual compounds was analyzed initially using the software package Grapher (Golden Software, Ltd.). Grapher was used to aid in describing the mathematical correlation between the mitochondrial respiratory inhibition and the concentration of compound under study. This program enables the graphing of data and proposal of possible best fit lines and curves including linear, logarithmic, exponential, cubic spline, power law, and various polynomial functions. This allows the generation of

possible models which may be further analyzed using regression analysis.

Regression analysis was performed using the mainframe version of SAS through the Oklahoma State University computer center. Possible models for each compound suggested by Grapher were analyzed for their ability to accurately describe the data with the fewest factors, the least error variance, and the highest correlation coefficients. Regression models which contained high correlation coefficients as well as fewer factors were chosen to describe the data. Once a model for each compound was obtained it was incorporated into a single equation based on the nature of the interactions of the chemicals in mixture to yield a single predictive equation which corresponds to the additive, synergistic, or antagonistic nature of the mixture.

CHAPTER IV

RESULTS

Individual and Mixture Effects

Individual Compounds

NADH oxidase activity was measured to determine the total activity of Complexes I, III, and IV of the respiratory chain.

Naphthalene (Figure 2), though assayed by Harmon and Sanborn (1982) was reassayed in this experiment and NADH oxidase activity was found to be inhibited between concentrations of 12.0 uM and 216.0 uM (1.54-27.69 ppm). Inhibition of respiration in the presence of naphthalene ranged from 13.70 % at 12.0 uM to 69.46 % at 216 uM. The EC_{50} (concentration which inhibits 50 % of respiration) for naphthalene was determined to be 15 ppm or 117 uM (Table I).

Acenaphthene (Figure 3) was tested at concentrations between 5.0 uM and 25.0 uM (0.769-3.85 ppm). Inhibition of mitochondrial respiration varied from 16.44 % at 5.0 uM to 47.45 % at 25.0 uM. The EC_{50} for acenaphthene corresponds to approximately 3.90 ppm or 25.3 uM (Table I). Acenaphthene was not assayed at concentrations higher than 3.85 ppm because its water solubility maximum is 3.93 ppm.

Figure 2. Inhibition of mitochondrial respiration by naphthalene plotting concentration of naphthalene vs. percent inhibition. A 50 percent inhibition occurs at a concentration of 15 ppm or 117 uM naphthalene. Equation of best fit determined by regression on a 95 percent confidence interval is: $y=.20\ (x) + 4.90\ (\ln x)$. Where y=percent inhibition and x=concentration of naphthalene (uM).

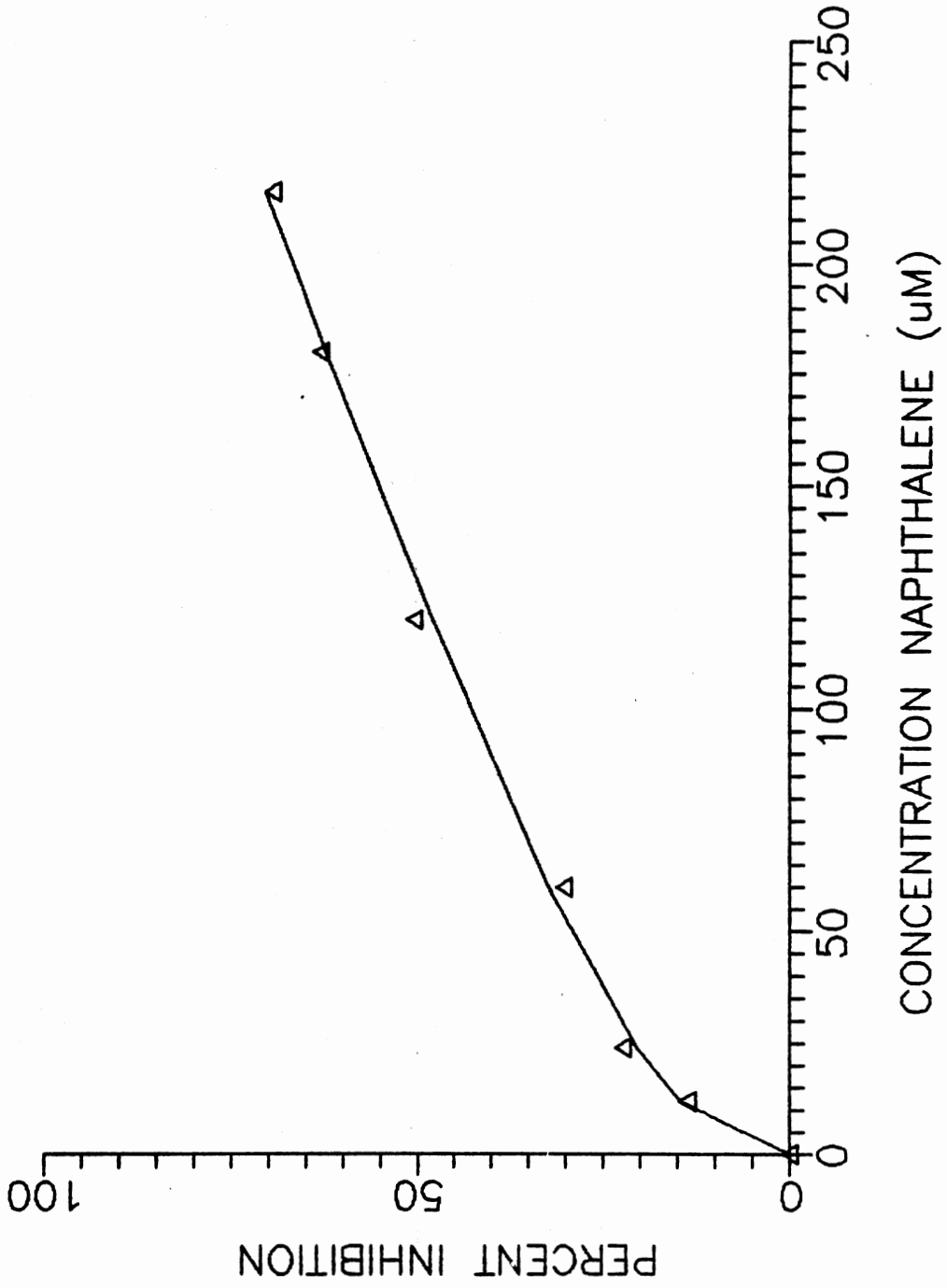
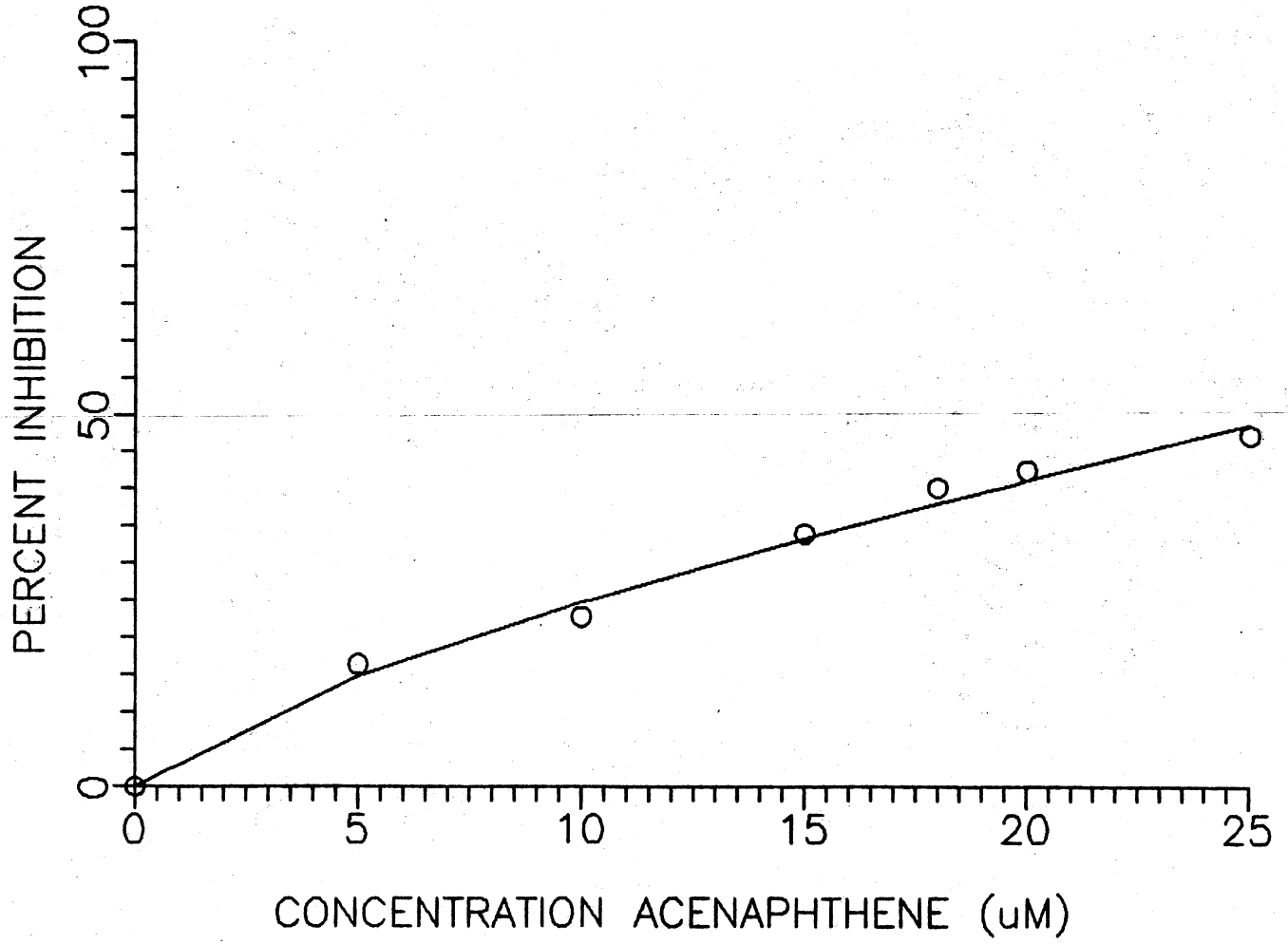

PERCENT INHIBITION
0
50
100
0
50
100
150
200
250
CONCENTRATION NAPHTHALENE (uM)

TABLE I

MEAN EFFECTIVE CONCENTRATIONS (EC_{50}'s) OF NAPHTHALENE ACENAPHTHENE, 1-CHLORONAPHTHALENE, AND BENZENE ON MITOCHONDRIAL RESPIRATION

COMPOUND	EC_{50} (ppm)
1-CHLORONAPHTHALENE	3.8
ACENAPHTHENE	3.9
NAPHTHALENE	15.0
BENZENE	525.0

Figure 3. Inhibition of mitochondrial respiration by acenaphthene plotting concentration of acenaphthene vs. percent inhibition. A 50 percent inhibition occurs at a concentration of 3.9 or 25.3 uM acenaphthene. Equation of best fit determined by regression on a 95 percent confidence interval is: $y=1.2\ (x) + 5.0\ (\ln x)$. Where y=percent inhibition and x=concentration of acenaphthene (uM).


PERCENT INHIBITION
100
50
0
0
5
10
15
20
25
CONCENTRATION ACENAPHTHENE (uM)

Benzene (Figure 4) was assayed at concentrations between 2.28 mM and 15.22 mM (178.3-1188.68 ppm). Respiratory inhibition varied from 9.29 % at 2.28 mM to 78.26 % at 15.22 mM. The EC_{50} for benzene based on these results was determined to be 525 ppm or 6.7 mM (Table I).

1-Chloronaphthalene (Figure 5) was tested at concentrations from 4.67 uM to 93.33 uM (0.769-15.38 ppm). Inhibition of mitochondrial respiration varied from 18.45 % at 4.67 uM to 92.45 % at 93.33 uM. The EC_{50} for 1-chloronaphthalene was calculated to be 3.8 ppm or 18.40 uM (Table I).

Additional Electron Transport Reactions

Partial electron transport reactions (Table II) were performed to determine a site of action for the four compounds tested. As mentioned above, the initial assays used NADH as the substrate to encompass complexes I, III, & IV of the electron transport chain. Succinate oxidase activity which measures the activity of complexes II, III, & IV was measured using succinate as substrate. No effect or only a slight inhibition of activity (less than 10 %) was observed for all four compounds. Cytochrome c oxidase activity (Table II), using ascorbate + TMPD as reductant to measure the activity of complex IV, was also not inhibited more than 3 % by any of the test compounds.

Complex I was further analyzed measuring NADH-quinone reductase activity to encompass all of complex I, quinol oxidase activity to measure that portion of the respiratory

Figure 4. Inhibition of mitochondrial respiration by benzene plotting concentration vs. percent inhibition. A 50 percent inhibition occurs at a concentration of 525 ppm or 6.7 mM benzene. Equation of best fit determined by regression on 95 percent confidence interval is: $y=3.0\ (x) + 10.0\ (\ln x)$. Where y=percent inhibition and x=concentration of benzene (mM).

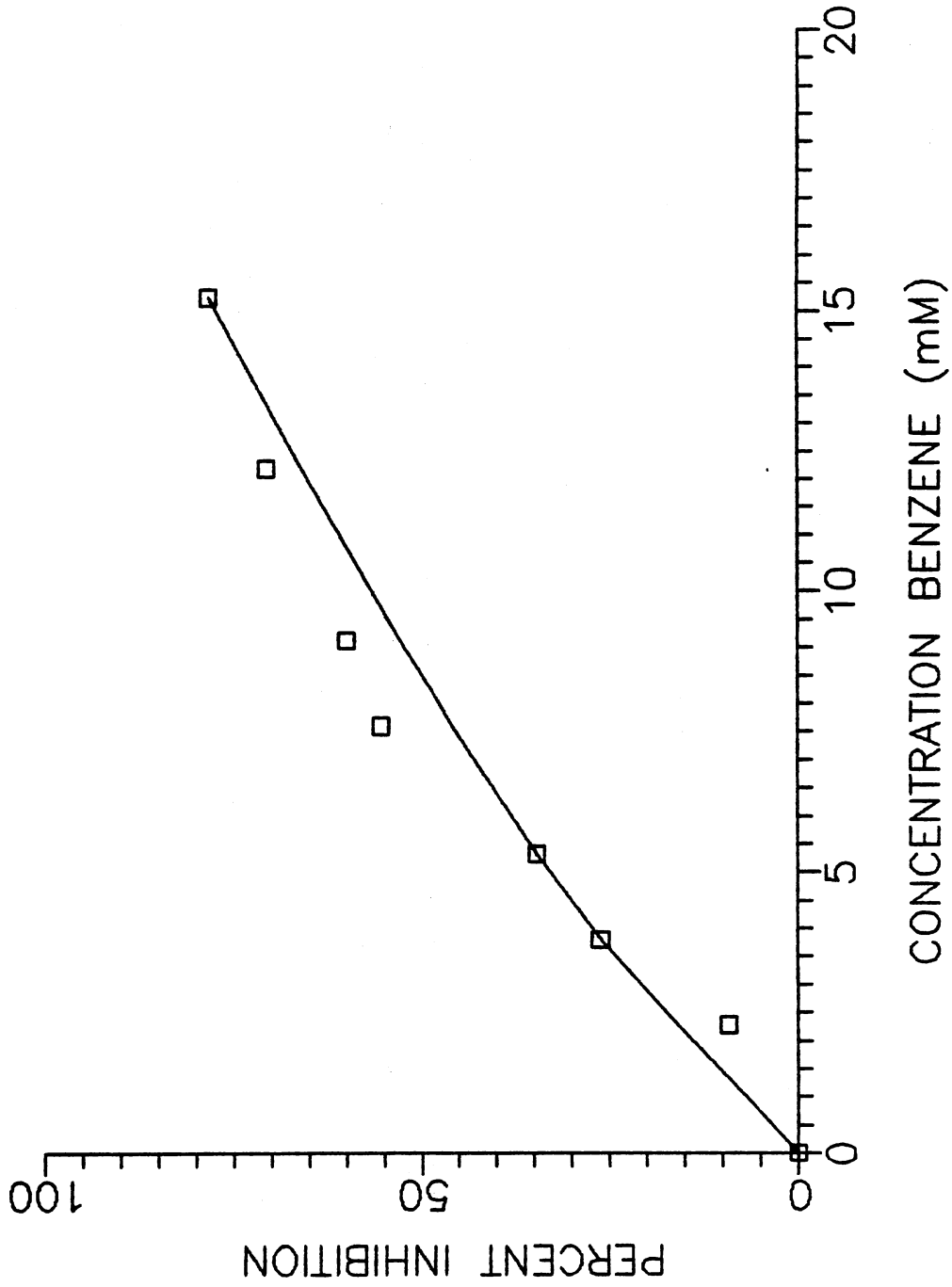

PERCENT INHIBITION
0
50
100
CONCENTRATION BENZENE (mM)
0
5
10
15
20

Figure 5. Inhibition of mitochondrial respiration by 1-chloronaphthalene plotting concentration vs. percent inhibition. A 50 percent inhibition occurs at a concentration of 3.8 ppm or 18.4 uM 1-chloronaphthalene. Equation of best fit determined by regression of a 95 percent confidence interval is: $y = 1.8(x) + 5.1(\ln x) - .012\ (x)$. Where y=percent inhibition and x=concentration of 1-chloronaphthalene (uM).

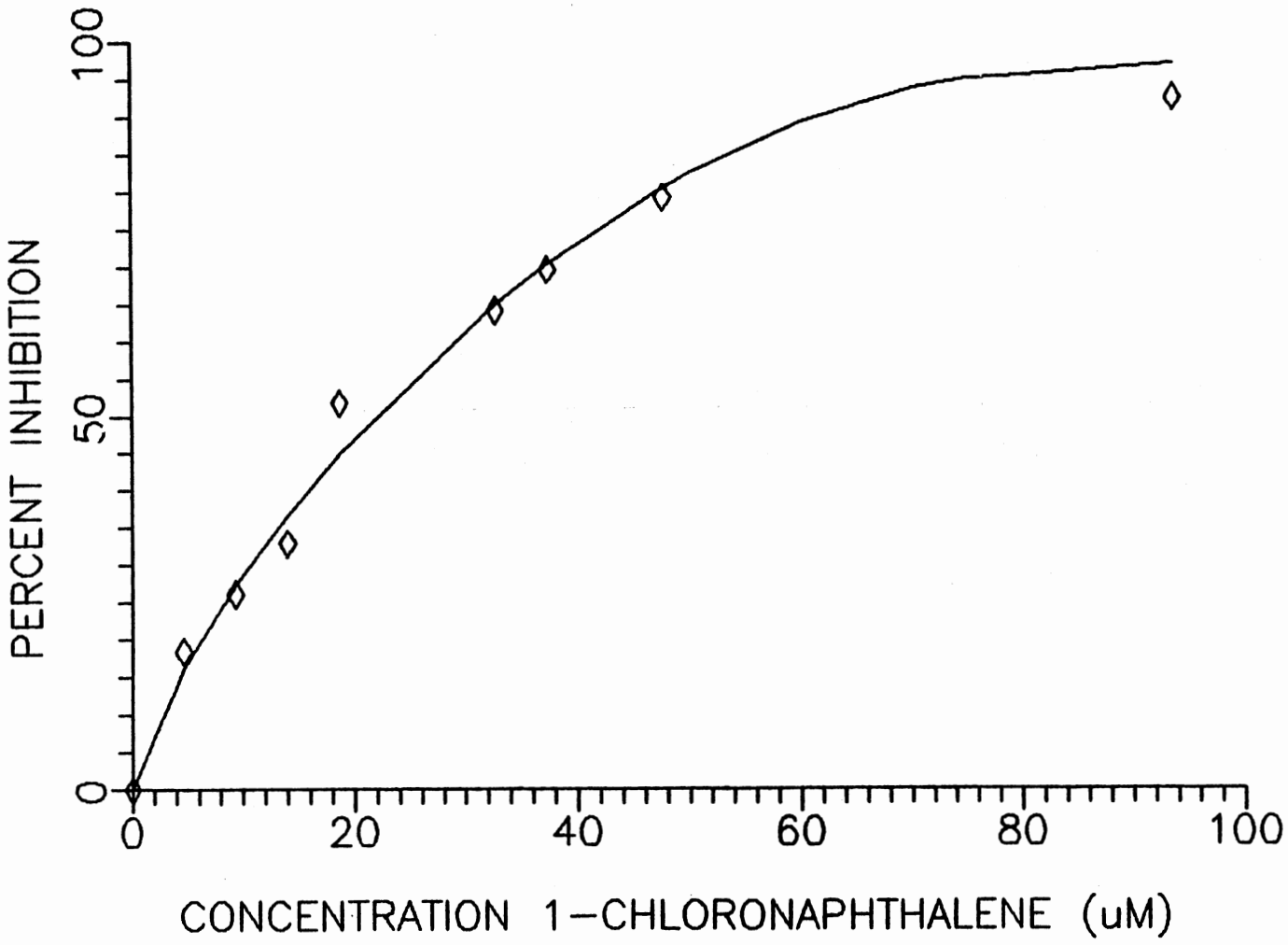

PERCENT INHIBITION
0
50
100
0
20
40
60
80
100
CONCENTRATION 1-CHLORONAPHTHALENE (uM)

TABLE II

EFFECT OF NAPHTHALENE, ACENAPHTHENE, BENZENE, AND 1-CHLORONAPHTHALENE ON ELECTRON TRANSPORT REACTIONS

ENZYME ASSAY	TYPICAL CONTROL RATE (nmoles O_2/min /mg/protein)	PERCENT INHIBITION BY NAP.	ACENAP.	BENZ.	1-CHLORNAP.
NADH->O_2	200-600	50.0	50.0	50.0	50.0
SUCCINATE->O_2	200-500	0-10[a]	5.4	4.2	9.2
CYTOCHROME c ->O_2	150-300	0.0	2.9	0.0	1.1
NADH-UBIQUINONE	100-250[b]	46.0	51.0	49.0	45.0
QUINOL->O_2	150-300	50.0	50.0	49.4	52.5
DUROQUINOL->O_2	200-700	0.0	1.2	2.6	0.0

NAPHTHALENE CONCENTRATION=15 ppm (117 uM)

ACENAPHTHENE CONCENTRATION=3.9 ppm (25.3 uM)

BENZENE CONCENTRATION=525 ppm (6.7 uM)

1-CHLORONAPHTHALENE CONCENTRATION=3.8 ppm (18.4 uM)

[a]STIMULATION OF ACTIVITY

[b]nMOLES ACCEPTOR/MIN/MG PROTEIN

chain from coenzyme Q to oxygen, and duroquinol oxidase activity to measure from the oxygen side of coenzyme Q to oxygen (Hare and Crane, 1971). NADH-quinone reductase was inhibited by all four compounds (Table II) with the level of inhibition corresponding to that found in the NADH-oxidase assays. Quinol oxidase (Table II) was also inhibited by all four compounds, to an extent similar to that of NADH-oxidase and NADH-quinone reductase. Duroquinol oxidase (Table II) was not inhibited by any of the compounds tested. This indicates a respiratory inhibition occurring at the level of coenzyme Q for all of the compounds tested.

Ultraviolet Spectrum of Coenzyme Q

Coenzyme Q has a single absorbance maximum at 275 nm in the ultraviolet range (Figures 6-8). Struble and Harmon (1983) previously demonstrated the alteration of this spectrum in the presence of naphthalene. At molar ratios above 175 naphthalene/Q two absorbance peaks are observed, one at 273 nm and one at 279 nm. In the presence of 35 ppm acenaphthene, sufficient to yield a molar ratio of 118 acenaphthene/Q (Figure 6) two absorbance maxima for coenzyme Q are observed at 263 nm and 275 nm. The 275 nm peak is non-symmetrical. In the presence of 30 ppm 1-chloronaphthalene which yields a molar ratio of 95 1-chloronaphthalene/Q (Figure 7) three absorbance maxima for coenzyme are observed at 257 nm, 270 nm, and 279 nm. Coenzyme Q in the presence of benzene at concentrations of 1545 ppm and 7726 ppm suffi-

Figure 6. Ultraviolet absorbance spectrum of ubiquinone in the presence of and absence of acenaphthene.

1. Absolute spectrum of 1.93×10^{-6} M ubiquinone in ethanol.
2. Difference spectrum of 1.93×10^{-6} M ubiquinone in 35 ppm acenaphthene minus 35 ppm acenaphthene ($\frac{\text{Acenaphthene}}{Q}$ = 118).

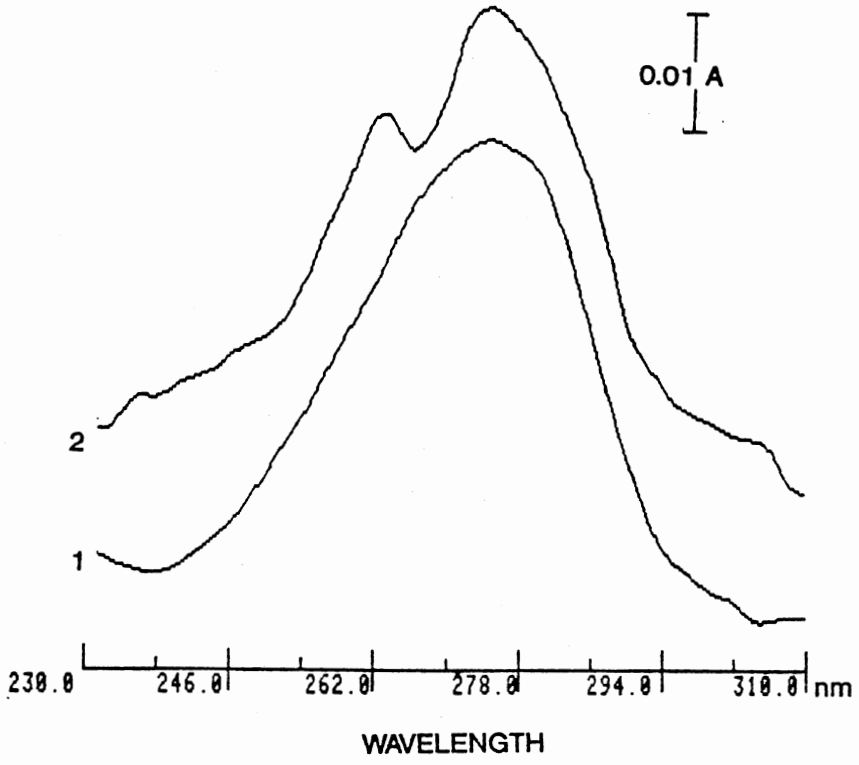

0.01 A
2
1
230.0
246.0
262.0
278.0
294.0
310.0
nm
WAVELENGTH

Figure 7. Ultraviolet absorbance spectrum of ubiquinone in the presence and absence of 1-chloronaphthalene.

1. Absolute spectrum of 1.93×10^{-6} M ubiquinone in ethanol.
2. Difference spectrum of 1.93×10^{-6} M ubiquinone in 30 ppm 1-chloronaphthalene minus 30 ppm 1-chloronaphthalene ($\frac{\text{1-chloronaphthalene}}{Q}$ = 95).

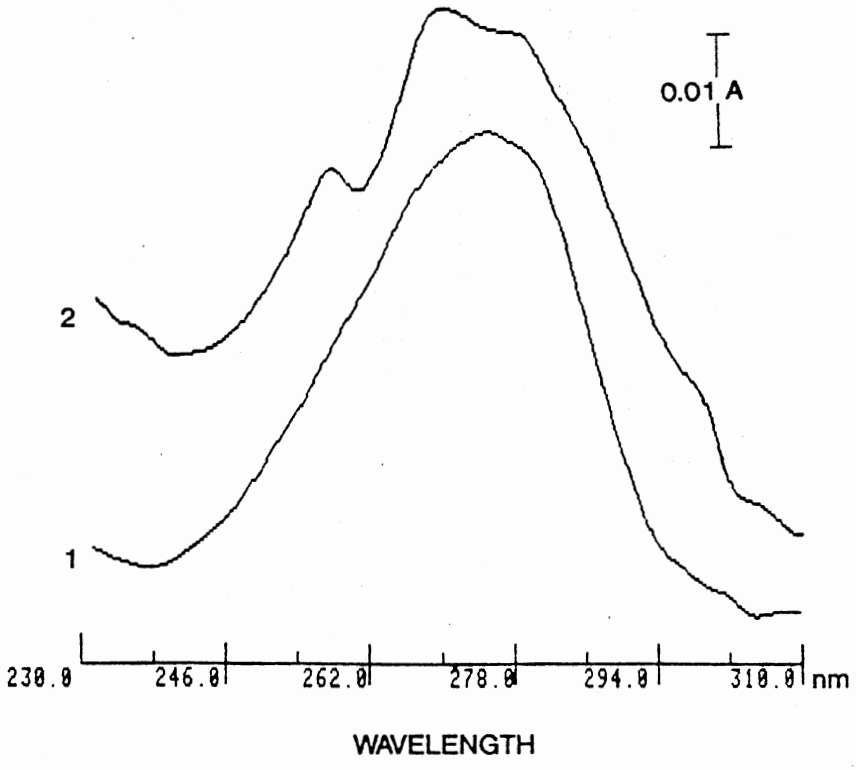
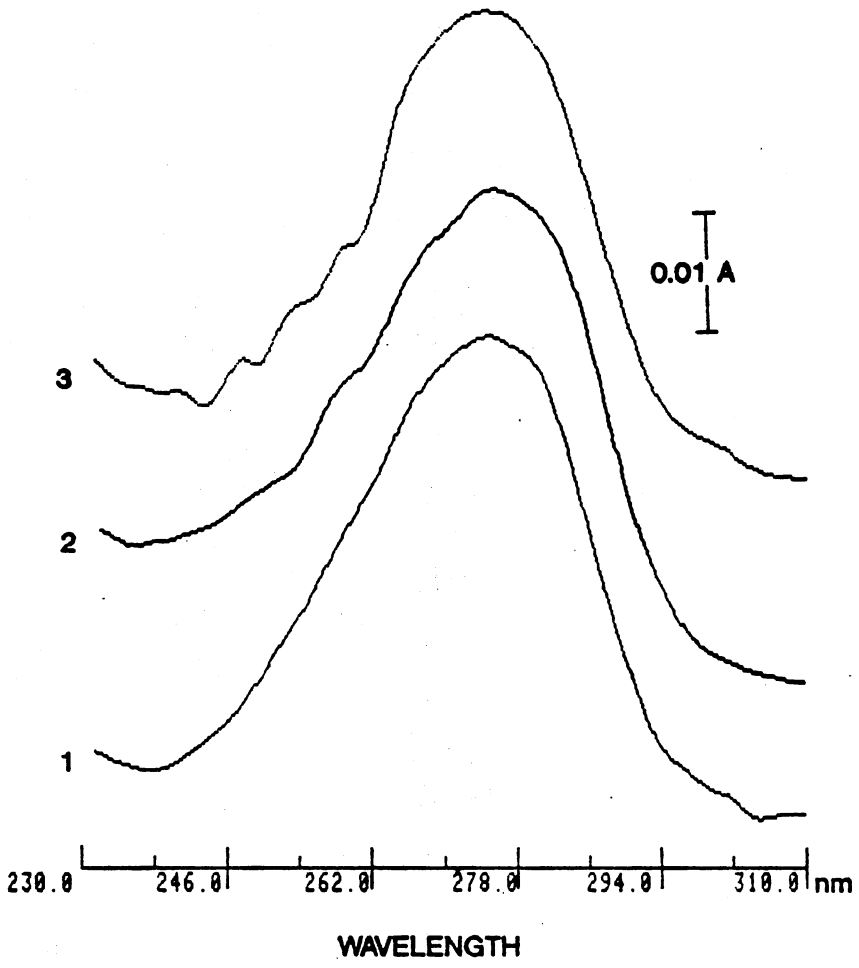

0.01 A
2
1
230.0
246.0
262.0
278.0
294.0
310.0
nm
WAVELENGTH

Figure 8. Ultraviolet absorbance spectrum of ubiquinone in the presence and absence of benzene.

1. Absolute spectrum of 1.93×10^{-6} M ubiquinone in ethanol.
2. Difference spectrum of 1.93×10^{-6} M ubiquinone in 1545 ppm benzene minus 1545 ppm benzene ($\frac{\text{Benzene}}{Q}$ = 10,252).
3. Difference spectrum of 1.93×10^{-6} M ubiquinone in 7726 ppm benzene minus 7726 ppm benzene ($\frac{\text{Benzene}}{Q}$ = 51,261).


0.01 A
3
2
1
230.0
246.0
262.0
278.0
294.0
310.0
nm
WAVELENGTH

cient to yield a molar ratios of 10,252 benzene/Q and 51,261 benzene/Q respectively has only one absorbance peak at 274 nm (Figure 8).

Mixtures

Mixtures of two to four compounds were analyzed using NADH as substrate since NADH oxidase activity was inhibited by all four of the compounds individually. A two chemical mixture containing acenaphthene and naphthalene (Table III) inhibited NADH oxidase activity additively. Therefore, the sum of the individual inhibitions of the compounds in the mixture was equal to the inhibition caused by the complete mixture. A three chemical mixture containing naphthalene, acenaphthene, and benzene (Table IV) was also additive.

Addition of 1-chloronaphthalene to the three chemical mixture yielded a four chemical mixture which also acted additively (Table V). The smallest respiratory inhibition observed for the four compound mixture, though additive, was near 50 percent since all compounds were present at acutely toxic concentrations (with their sum inhibition approximately 50 percent). Inhibition below 50 percent with the two and three chemical mixtures was also additive (see Chapter V).

TABLE III

COMPARISON OF AN EXPECTED ADDITIVE RESPIRATORY INHIBITION WITH ACTUAL INHIBITION USING A TWO COMPOUND MIXTURE

*(HOLDING ACENAPHTHENE CONSTANT AT 5uM AND VARYING NAPHTHALENE)

CONCENTRATION NAPHTHALENE (uM)	EXPECTED INHIBITION IF ADDITIVE (%)	ACTUAL INHIBITION (%)	ABSOLUTE DIFFERENCE (%)
12	30.14	28.95	1.19
24	38.74	33.33	5.41
60	46.74	47.15	0.41
120	66.89	65.00	1.89
180	79.53	75.93	3.60
216	85.91	82.46	3.45

*NOTE: ACENAPHTHENE WAS ALSO VARIED. RESULTS WERE ALSO ADDITIVE

TABLE IV

COMPARISON OF AN EXPECTED ADDITIVE RESPIRATORY INHIBITION WITH ACTUAL INHIBITION USING A THREE COMPOUND MIXTURE
* (HOLDING ACENAPHTHENE AT 5 uM AND BENZENE AT 2.28 mM)

CONCENTRATION NAPHTHALENE (uM)	EXPECTED INHIBITION IF ADDITIVE (%)	ACTUAL INHIBITION (%)	ABSOLUTE DIFFERENCE (%)
12	39.43	40.85	1.42
24	48.03	47.48	0.55
60	56.03	57.95	1.92
120	76.18	75.45	0.73
180	88.12	85.92	2.90
216	95.20	90.32	4.88

* NOTE: OTHER COMPOUNDS WERE ALSO VARIED. RESULTS WERE ADDITIVE FOR ALL TESTS.

TABLE V

COMPARISON OF AN EXPECTED ADDITIVE RESPIRATORY INHIBITION WITH ACTUAL INHIBITION USING A FOUR COMPOUND MIXTURE
* (HOLDING ACENAPHTHENE AT 5 uM, BENZENE AT 2.28 mM, AND 1-CHLORONAPHTHALENE AT 4.67 uM)

CONCENTRATION NAPHTHALENE (uM)	EXPECTED INHIBITION IF ADDITIVE (%)	ACTUAL INHIBITION (%)	ABSOLUTE DIFFERENCE (%)
6	54.16	50.92	3.24
12	57.88	55.71	2.17
18	61.94	62.76	0.82
24	66.48	66.31	0.17
30	67.85	69.82	1.97
60	74.48	75.86	1.38

* NOTE: OTHER COMPOUNDS WERE ALSO VARIED. RESULTS WERE ADDITIVE FOR ALL TESTS.

CHAPTER V

DISCUSSION

Toxicity of the Individual Compounds

The magnitude of toxicity of the compounds tested in this study increases in the following order based on $EC_{50's}$ from the mitochondrial bioassays: benzene (525 ppm) < naphthalene (15 ppm) < acenaphthene (3.9 ppm) < 1-chloronaphthalene (3.8 ppm). These compounds, as mentioned in Chapter II, belong to a class of relatively nonreactive, non-ionizable compounds generally thought to exert their acute toxicity through a common, nonspecific mode of action referred to as narcosis (Deneer et al., 1988; Broderius and Kahl, 1985; DeWolf et al., 1988). The acute effect of these compounds is said to be governed only by their hydrophobicity (Konemann, 1981; Hermans et al., 1984a; Vieth, 1983). The toxicity of the compounds in this work increase with increasing log octanol-water partition coefficients (log P's). A comparison between log P and the $EC_{50's}$ for the test compounds is listed in Table VI. The log P of acenaphthene was not found in the literature. Acenaphthene ($C_{12}H_{10}$) is a slightly larger aromatic compound that naphthalene ($C_{10}H_8$ and log P=3.30) and yet smaller than fluorene ($C_{13}H_{10}$ and log P=4.18). Therefore, since log P increases

TABLE VI

LOG OF OCTANOL/WATER PARTITION COEFFICIENT (LOG P)

COMPOUND	PARTITION COEFFICIENT
BENZENE	2.13
NAPHTHALENE	3.30
ACENAPHTHENE	3.50-4.00*
1-CHLORONAPHTHALENE	4.08

*Acenaphthene ($C_{12}H_{10}$) partition coefficient not found cited in literature. Therefore log P is estimated, based on structure, to be between 3.5 and 4.0 knowing that acenaphthene is a larger aromatic than naphthalene ($C_{10}H_8$ and log P = 3.30) and a smaller aromatic (based on carbon content) than fluorene ($C_{13}H_{10}$ and log P = 4.18).

with increasing carbon content the log P for acenaphthene was estimated to be between 3.5 and 4.0.

Hydrophobicity is believed to be correlated to the ability of a compound to partition into biological membranes such as the mitochondrial inner membrane. Log P serves as a measure of hydrophobicity and is established by mixing a compound in 1-octanol and water then measuring the relative distribution of the compound between the two phases. The more hydrophobic the compound the more it will partition into the organic phase.

Interaction of Test Compounds In Vitro

All four compounds analyzed in this work inhibit NADH oxidase, NADH-ubiquinone reductase, and quinol oxidase enzyme systems, yet lack a significant effect on succinate oxidase, cytochrome c oxidase, and duroquinol oxidase systems (Table II). This points to an inhibition of the mitochondrial respiratory chain at the level of coenzyme Q. This is the same site of action as previously reported by Harmon and Sanborn (1982) for naphthalene. Since complexes I and II both transfer electrons to ubiquinone it is interesting that NADH oxidase activity is inhibited and succinate oxidase activity is not. Quinones, such as ubiquinone, have an isoprenoid side chain attached to them which is not involved in electron transport, but affects the interaction between coenzyme Q and the NADH or succinate dehydrogenases (Folkers, 1975). Two separate quinone pools appear to be

present in mitochondria which contain both long-chain or short-chain Q homologs. Those quinones which have shorter isoprenoid chains (8 or less isoprenoid units) appear to be relatively ineffective at transferring electrons from Complex I to Complex III. Complex II, however appears to be less specific and electron transfer to Complex III will occur in the presence of short or long chain quinones (Folkers, 1975). The Q_{10} homolog of coenzyme Q is indicated as the site of action for the compounds under study (see below). Inhibition of NADH oxidase but not succinate oxidase in the presence of "Q_{10}" inhibitors is likely the result of the specific requirement of long-chain quinones by Complex I. Succinate oxidase activity is left unaffected since electron transfer between complexes II-III still occurs via short-chain quinone homologs.

Struble and Harmon (1983) suggested that the interaction of naphthalene with ubiquinone, which interfered with the coenzyme's reduction, was either a specific interaction with ubiquinone itself, an interaction with Q-binding protein discussed by Yu et al. (1977), or both. Struble and Harmon (1983) were able to demonstrate a specific interaction between naphthalene and the ubiquinone as given by the splitting of the single ultraviolet (275 nm) absorbance maximum of coenzyme Q into two absorbance maxima; 273 and 279 nm. This perturbation of ubiquinone was observed at molar ratios above 175 naphthalene/Q. Intact bovine heart mitochondria have been reported to contain ubiquinone at a

concentration between 2.9 nmoles/mg protein (Crane et al., 1957) and 3.5 nmoles/mg protein (Huang and Lee, 1975). Therefore, an EC_{50} of 10 ppm (78 uM) naphthalene would correspond to a molar ratio of naphthalene to ubiquinone of 379 to 460 and is more than twice the concentration required to perturb the spectrum of the quinone. The spectrum of duroquinone (tetramethylbenzoquinone) was not affected by the presence of naphthalene nor was duroquinol oxidation in intact mitochondria (Struble and Harmon, 1983). The concentrations of compounds used in this study were chosen to yield a molar ratio of perturbant compound to coenzyme Q equivalent to, or below, that which is given by the ratio of the EC_{50} of the compound to Q in intact mitochondria.

Acenaphthene affects the ultraviolet spectrum of ubiquinone a ratios above 118 acenaphthene to ubiquinone (Figure 6). The 3.8 ppm (23.5 uM) EC_{50} of acenaphthene required to inhibit respiration would correspond to molar ratios between 123 and 149 based on the ubiquinone content of intact mitochondria described above. Acenaphthene causes a splitting of the ubiquinone peak at 275 nm resulting in absorbance maxima at 275 nm and 263 nm. The 275 nm peak is also a nonsymmetrical peak indicating another possible perturbation (though undistinguishable) of the spectrum of ubiquinone. The 12 nm separation of the two maxima is well within the resolution of the spectrophotometer with a slit half-bandwidth of 1 nm (1 nm resolution). The observed maxima are not dilution artifacts which would have corre-

sponded to changes in the 243 nm, 279 nm, and 288 nm maxima of acenaphthene.

1-Chloronaphthalene also causes a splitting in the absorbance maxima of ubiquinone (Figure 7). In the presence of 1-chloronaphthalene at molar ratios above 75 1-chloronaphthalene to coenzyme Q three absorbance maxima are observed at 257 nm, 270 nm, and 278.6 nm. The EC_{50} of 1-chloronaphthalene (3.8 ppm or 18.4 uM), based on oxygen consumption assays, corresponds to molar ratios between 90 and 108 1-chloronaphthalene to ubiquinone in intact mitochondria. Data for the perturbation of ubiquinone by 1-chloronaphthalene (Figure 7) is presented at a molar ratio of 95 1-chloronaphthalene to ubiquinone. The three absorbance maxima observed are all within the resolution of the spectrophotometer (1 nm), and dilution artifacts of 1-chloronaphthalene would have corresponded to the wavelengths 273 nm, 283 nm, and 291 nm of the 1-chloronaphthalene ultraviolet spectrum.

Benzene, even at molar ratios as high as 10,252 and 55,261 benzene to ubiquinone, failed to cause either shifting or splitting in the spectrum of ubiquinone (Figure 8). Only a single absorbance peak at 274 nm is observed with the 1 nm difference between absolute ubiquinone and ubiquinone in the presence of benzene not within the resolution of the spectrophotometer. The EC_{50} of the benzene mitochondrial respiration assays (525 ppm or 6.7 mM) corresponds to a ratio between 32,560 and 39,410 benzene to ubiquinone in

intact mitochondria. Dilution artifacts of benzene and not peaks corresponding to ubiquinone are present and would account for absorbance peaks at 239 nm, 243 nm, 249 nm, and 254 nm wavelengths in the ultraviolet spectrum of benzene. These artifacts were present, most notably in the 55,261 ppm benzene and ubiquinone spectrum (Figure 8, trace 3) due to the extremely high concentration of benzene and the inability of the machine to fully subtract out the corresponding absorbance maxima.

Staples and Szwarc (1970) have previously observed shifts in the wavelength maxima of quinone compounds (phenanthrenequinone) due to a dimerization of the quinones via a pi-electron interaction (there was no evidence to suggest bond formation). Alteration in the environment of quinone and porphyrin compounds also affects their absorbance spectrum (Schleyer et al. 1971). Phillips et al. (1969) demonstrated solvent dependent changes in the absorbance spectrum of 2,6-dimethylbenzoquinone with the replacement ethanol for methanol inducing a 3 nm bathochromic shift (to a higher wavelength).

The spectrum of ubiquinone undergoes a 4 nm bathochromic shift in the presence of naphthalene, a 12 nm hypsochromic shift in the presence of acenaphthene, and both a 4 nm bathochromic and 18 nm hypsochromic shift in the presence of 1-chloronaphthalene. All of these alterations are well within the resolution of the spectrophotometer and do not correspond, as noted above, to artifacts from the test

compounds. Struble and Harmon (1983) speculated that this change in the spectrum of ubiquinone, in the presence of naphthalene, could be the result of perturbations in the pi-electron cloud of the quinone or in its environment. The hypothesis that a change in the polar environment of the quinone would induce an alteration in the quinone spectrum is well supported in the literature (Simmons, 1979; Staple and Szwarc, 1970; Phillips et al. 1969). Acenaphthene, and 1-chloronaphthalene, due to their structural similarity with naphthalene, would also be expected to affect either the pi-electron cloud of ubiquinone or its environment.

Since coenzyme Q is an essential component of the mitochondrial electron transfer chain, shuttling electrons between complexes I-III and II-III, alteration in its physical state may effect its ability to bind to Q-binding proteins (Yu et al., 1977; Struble and Harmon, 1983) or participate in essential redox reactions, and thus, transfer electrons. Since respiration is ultimately dependent on electron transfer it may be inhibited by alterations of ubiquinone. Benzene failed to affect the spectrum of ubiquinone, yet does effect mitochondrial respiration at the level of coenzyme Q. This effect, however is at a very high concentration of benzene; we hypothesize here that the single aromatic ring of the benzene molecule is not capable of causing the alteration of the ubiquinone spectrum observed with the polynuclear aromatic hydrocarbons. This could be due to the lower hydrophobicity of benzene or

because benzene is too small to meet some spatial requirement to inhibit ubiquinone.

The compounds tested in this study, though obviously affecting mitochondrial function *in vitro*, should not be implied to have this mitochondrial effect as their sole mode of action. In this work the mitochondrion is simply being used as a tool to assess the interaction of these compounds *in vitro* so that speculation may be made regarding their effects in mixtures and *in vivo*. However, the fact that all four aromatic hydrocarbons act as mitochondrial respiratory inhibitors may suggest an *in vivo* effect of this class of compounds as noted by Harmon and Sanborn (1982).

The Interaction of the Mixtures

Mitochondrial respiration was inhibited by mixtures of 2-4 of the test compounds with the overall toxicity of the mixtures not appreciably different than that expected based on the sum of the toxicities of the mixture components (Tables III-V). This finding is supported in the literature regarding previous *in vivo* testing. As mentioned in Chapter II, acute toxicity tests with *Daphnia magna* (Hermans et al., 1984a and 1984b) and the fathead minnow (Broderius and Kahl, 1985) using mixtures of between 5 and 50 compounds similar to those in this study were reported to act additively. The additivity of these mixtures was independent of the concentration of the constituents, such that a compound contributes to the overall toxicity of the mixture even if it is

present at a concentration below its "no effect level" (Deneer et al., 1988).

QSAR (Hermans et al., 1984a; DeWolf et al., 1988;) and CA (Deneer et al., 1988; Hermans et al. 1984b) models have been used predict the effects of additive mixtures. These models have been verified (DeWolf et al., 1988; Hermans et al., 1984a and 1984b) to describe additive mixture toxicity quite accurately. As discussed below, a model using regression analysis would also be useful for predicting the toxicity of additive mixtures.

Regression Models

Regression models were generated from data based on the correlation between respiratory inhibition and concentration for each of the individual compounds. Equations (models) to describe the effects of the individual compounds were chosen based on their ability to explain the variance within the data, high correlation coefficients, small error variance, and their simplicity related to the number of independent variable (compound concentration) factors (Table VII). Thus, equations were chosen which had the highest correlation for the fewest number of factors. The equations given by the regression (see legends of Figures 2-5) for naphthalene, acenaphthene, and benzene are based on the sum of a function of x and a function of the natural logarithm of x, where x is the concentration of compound. 1-chloronaphthalene has an additional x^2 term which lowered the

TABLE VII

COMPARISON OF CORRELATION OF POSSIBLE MODELS
ANALYZED WITH MULTIPLE REGRESSION

MODELS (y=)	CORRELATION (r =1.0=perfect)	COMMENTS
1) x	0.940[a] 0.989[b] 0.963[c] 0.883[d]	Fairly High but others better.
2) $\ln x$	0.960[a] 0.978[b] 0.953[c] 0.968[d]	Higher or not much difference, but 3) better.
3) $x + \ln x$	0.9973[a] 0.9970[b] 0.9757[c] 0.9864[d]	High correlation and few factors.
4) $x + \ln x + x^2$	0.9976[a] 0.9971[b] 0.9890[c] 0.9980[d]	Not significantly better vs. 3) but more factors. d better.
5) $x + \ln x + x^2 + x^{1/2}$	0.9978[a] 0.9980[b] 0.9890[c] 0.9977[d]	Not significantly better vs. 3) or 4) but more factors.

x^2 and $x^{1/2}$ factors did not have a significant effect on the correlation with compounds a-c yet made for a more complicated equation. For this reason equation 3 was chosen for these compounds. Compound d had very high error variance with 2 factor model therefore, 3 factor model chosen.

[a]Data corresponding to naphthalene
[b]Data corresponding to acenaphthene
[c]Data corresponding to benzene
[d]Data corresponding to 1-chloronaphthalene

y=percent inhibition and x=concentration of inhibitor

error variance of the equation significantly from 63 to 3. Therefore, the 3 factor model for 1-chloronapthalene best explains the data and had the highest correlation (Table VII). Accurate prediction of mixture toxicity comes from accurate prediction of the effects of each mixture component.

Modeling for the three and four chemical mixtures involves consideration of the interactions between the compounds and, in the cases of synergistic or antagonistic mixtures, the development of an appropriate interaction term(s). Since the interaction between all chemicals tested in this study is additive, a model may be taken from the sum of each of the individual equations which best described the effects of the single compounds (see legends of Figures 2-5). A summary of the steps for developing a regression equation to explain mixture data are given in Table VIII.

Prediction of toxicity of the three compound mixture is compared to actual mixture data in Figure 9. The model (which is the sum of the regression equations for naphthalene, acenaphthene, and benzene) is able to predict to within approximately 5 percent for all the points tested. Though only naphthalene was varied in this graph (for ease of representation) random mixtures as well as varying the concentrations of other compounds did not effect the additive nature of the mixture. Since all compounds were tested at toxic concentrations the lowest inhibition observed with the three chemical mixture was approximately 40 percent.

TABLE VIII

DETERMINATION OF BEST FIT MODEL FOR EACH COMPOUND AND FOUR COMPOUND MIXTURE

1. The data and possible models were analyzed using multiple regression and a model is determined for each compound under study (Figures 3-6).

2. The process started with a four factor model (Table VII) and then a stepwise elimination to find the simplest and most accurate model. The best equation is derived from the regression analysis and put in the form; $y=B_1 (x_1) + B_2 (\ln x_1)$ etc. , where y is the percent respiratory inhibition, x is the concentration of the compound, and the betas (B) are regression coefficients.

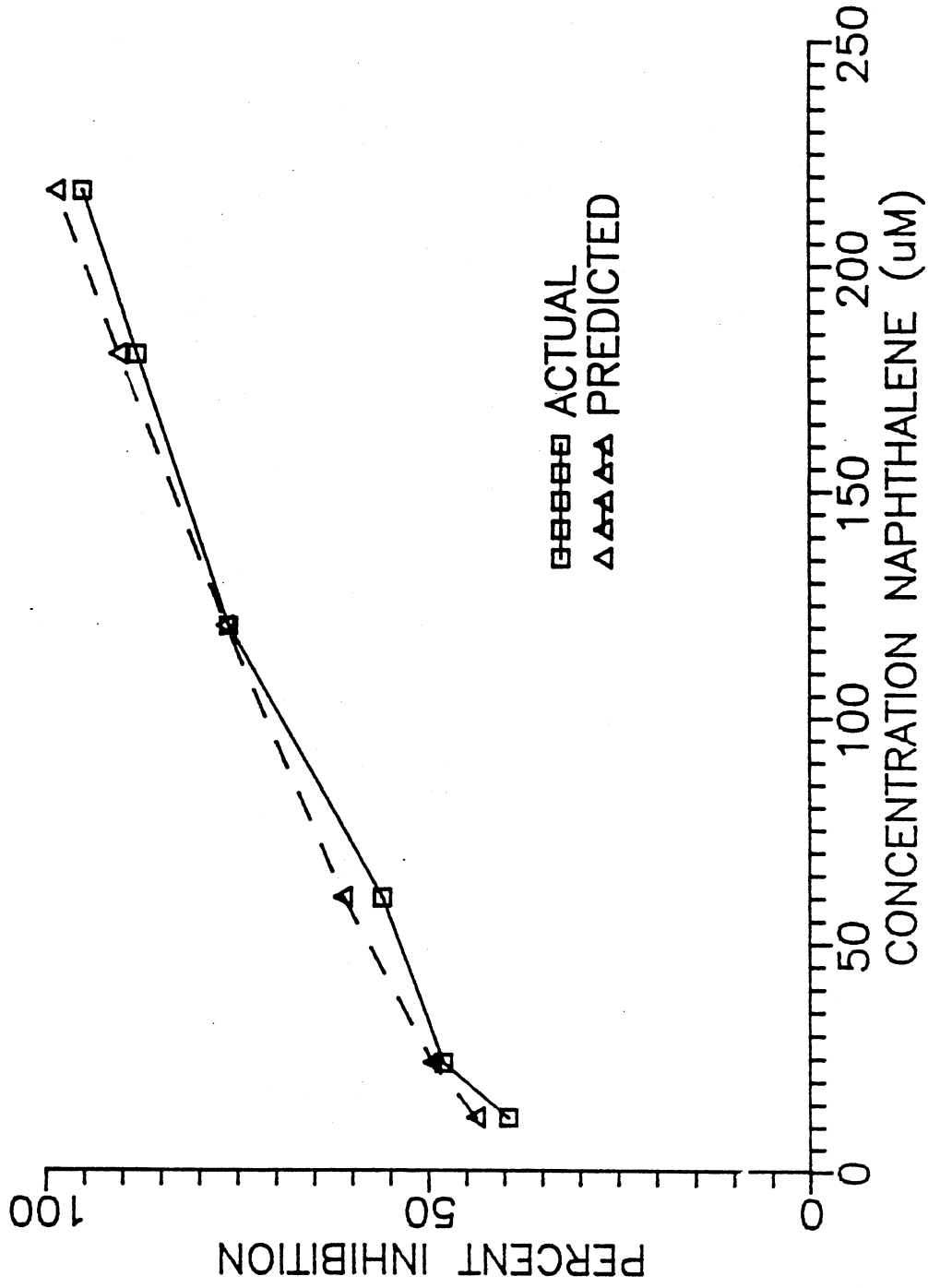
3. Possible interaction is determined from measuring effects of mixtures of two to four compounds (Tables III-V). If there is an interaction, other than additive, an interaction term statistically derived can be incorporated into the final model.

4. The final equation is derived to incorporate the equations of the individual compounds and any interaction between compounds in mixtures. For an additive four compound mixture :

$$Y=B_1 (x_1)+ B_2 (\ln x_1) + B_3 (x_2) + B_4(\ln x_2)+ B_5 (x_3) + B_6 (\ln x_3) + B_7 (x_4)+ B_8 (\ln x_4) + B_9 (x^2_4).$$

Where Y=inhibition of respiration caused by the mixture.

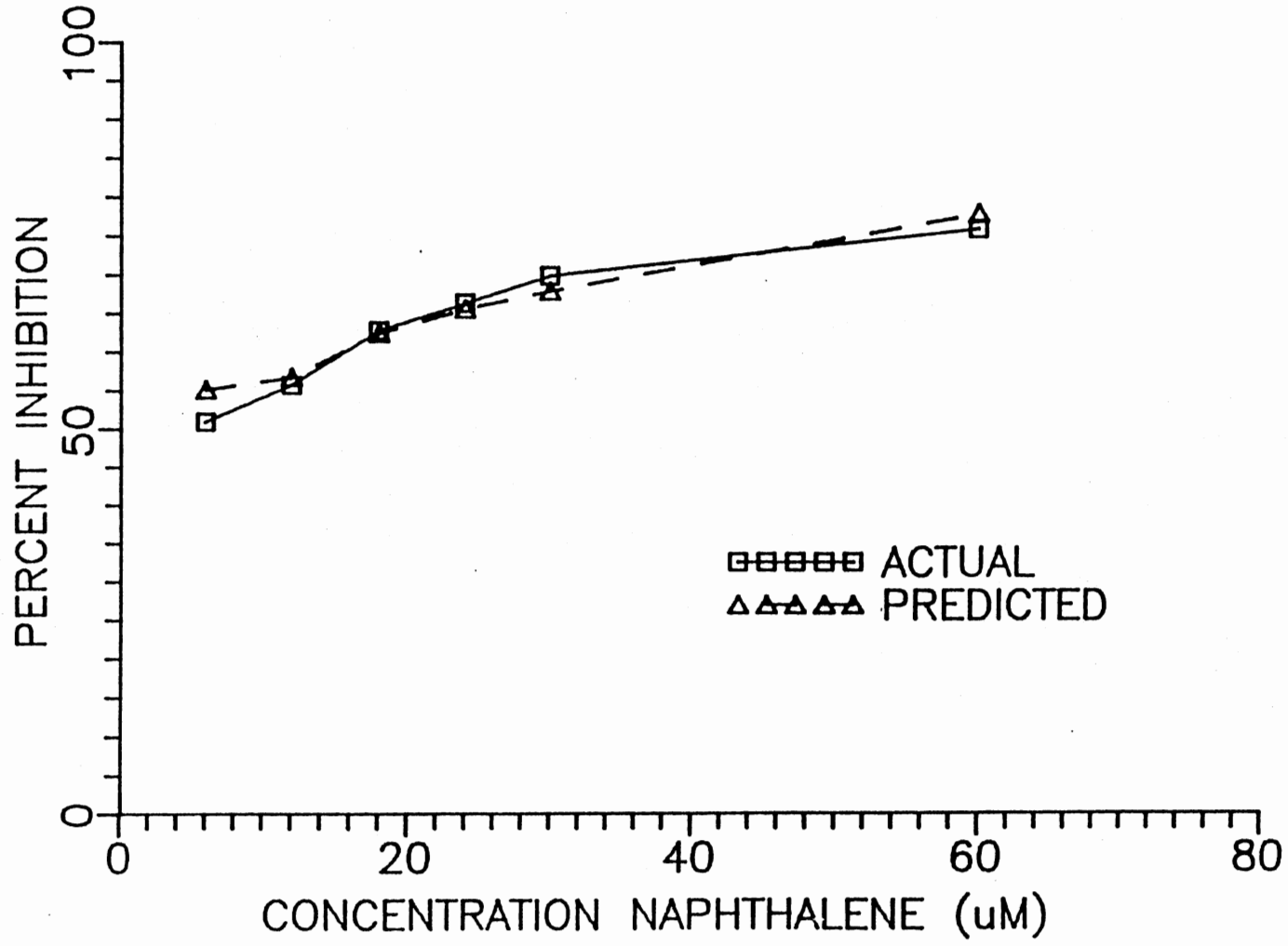
Figure 9. Comparison of predicted respiratory inhibitions from best fit equation vs. actual inhibitions from three compound mixtures. Acenaphthene was held constant at 5 uM, benzene at 2.28 mM, and naphthalene was varied. Equation of best fit determined by regression on a 95 percent confidence interval for an additive interaction is $Y=.20(x_1)+ 4.9(\ln x_1) + 1.2(x_2)+ 5.0(\ln x_2) + 3.0(x_3)+ 10.0(\ln x_3)$. Where Y=the toxicity of the mixture and x_1, x_2, and x_3=the concentrations of naphthalene, acenaphthene, and benzene respectively.


PERCENT INHIBITION
100
50
0
0
50
100
150
200
250
CONCENTRATION NAPHTHALENE (uM)
ACTUAL
PREDICTED

Data regarding the interaction of the compounds below their "no effect level" was not obtained in this study. However, the two chemical mixture (Table III) was also additive at levels of inhibition from 28 percent to 83 percent, and Deneer et al. (1988) has shown these types of compounds to act additively at low concentrations. Therefore, since these compounds are observed to be additive both *in vivo* and *in vitro* an additive interaction is still expected to occur at low concentrations with the *in vitro* mitochondrial assay.

Four compound additive mixtures were also modeled and the regression equation is given in the legend of Figure 10. Predictability from the regression equation with the four compound mixture was also quite good and less than 5 percent difference between the predicted toxicity and actual mixture toxicity was observed. Again, these tests were performed at acutely toxic concentrations of each of the test compounds, and thus, the lack of data mixture concentrations below 50 percent.

Figure 10. Comparision of predicted respiratory inhibitions generated from best fit equation vs. actual inhibitions from three compound mixtures. Acenaphthene was held constant at 5 uM, benzene at 2.28 mM, and 1-chloronaphthalene at 4.67 mM. Naphthalene was varied. Equation of best fit determined by multiple regression on a 95 percent confidence interval for an additive mixture is $Y=.20(x_1) + 4.9(\ln x_1) + 1.2\ (x_2) + 5.0(\ln x_2) + 3.0\ (x_3) + 10.0\ (\ln x_3) + 1.8(x_4) + 5.11\ (\ln x_4) - .012(x^2_4)$. Where Y=the overall toxicity of the four compound mixture and x_1, x_2, x_3, and x_4 are the concentrations of naphthalene, acenaphthene, benzene, and 1-chloronaphthalene respectively.


PERCENT INHIBITION
100
50
0
0
20
40
60
80
CONCENTRATION NAPHTHALENE (uM)
ACTUAL
PREDICTED

CHAPTER VI

CONCLUSIONS

General

The use of the *in vitro* mitochondrial respiration assay to analyze the interactions of hydrophobic aromatic compounds reveals three very important findings related to both the individual compounds and the effects in complex mixtures. First, the compounds were shown to all act at the same site (by the same mode) of action. The compounds were also found to act very specifically with coenzyme Q of the mitochondrial respiratory chain. Finally, mixtures of the four compounds acted additively, likely due to the fact that they have the same site of action. Each of these points will be discussed below.

Similar Site of Action

All four compounds tested in this study have been shown to have the same site of action. Naphthalene, acenaphthene, benzene, and 1-chloronaphthalene all inhibited NADH oxidase, quinol oxidase, and NADH-ubiquinone reductase, but failed to have an effect on succinate oxidase, cytochrome *c* oxidase, or duroquinol oxidase. As mentioned previously, this points to a respiratory inhibition occurring at coenzyme Q, the

electron transport component responsible for transferring electrons to Complex III of the respiratory chain. Previous studies with the anesthetic-type aromatic hydrocarbons (Hermans et al., 1984a and 1984b; Deneer et al., 1988; DeWolf et al., 1988; Broderius and Kahl, 1985) all make the assumption that these compounds have a similar mode of action. The *in vitro* mitochondrial and spectrophotometric assays used in this study verify the fact that at least with some of the simple aromatics (1-3 rings) they do indeed have a common mode of action, and thus this study complements previous *in vivo* studies which made this assumption. However, these studies also indicated that the compounds act via a nonspecific membrane perturbation and that this was the basis for their toxic effects. Described in the next section is the argument that these compounds may have a rather specific effect with a membrane component.

Specific Interaction at Coenzyme Q

As noted above, all four of the test compounds act to inhibit coenzyme Q of the mitochondrial respiratory chain. This interaction is not due to a membrane perturbation but rather a specific interaction with the coenzyme itself. If the inhibition of mitochondrial respiration was a generalized, non-specific membrane effect all of the enzyme sequences of the respiratory chain would likely be inhibited and not just those encompassing coenzyme Q (Table II). Furthermore, the perturbation of the ultraviolet spectrum of

ubiquinone by naphthalene, acenaphthene, and 1-chloronaphthalene (Figures 6-8) indicates a very specific interaction with the coenzyme itself, by these compounds. As noted in Chapter V, an alteration in the physical nature of the quinone (either its pi-electron cloud or by its environment) could affect its ability to bind a Q-binding protein or accept and donate electrons. Benzene, though it doesn't perturb coenzyme Q, still effects at the level of the coenzyme.

This finding does no agree with the general consensus regarding the membrane perturbing, "anesthetic" effect of this class of aromatic hydrocarbon. As noted in Chapter V, this study does not mean to discredit the possibility of membrane perturbation as the in vivo mode of action of these compounds. However, evidence from this study shows that these compounds have a very specific mode of action on mitochondria, and thus further studies in vivo are deemed necessary to establish if a rather specific action of these compounds exists in vivo and may be responsible for their observed toxic effect.

Additive Effects of Test Compounds on Mitochondrial Respiration

The fact that the four compounds tested in this study act additively also concurs with previous in vivo tests (Deneer et al., 1988; DeWolf et al., 1988; Broderius and Kahl et al., 1985), and indicates that this additive effect

is indeed the result of a similar mode of action. These compounds which all act at coenzyme Q simply increase the magnitude of the respiratory inhibition when present with other "Q inhibitors". Previous work (Deneer et al., 1988; Hermans et al., 1984a) with this same type of aromatic hydrocarbon predicted, based on either QSAR models (Hermans et al., 1984a) or concentration addition models (Broderius and Kahl, 1985), the additive toxic effect of between 5 and 50 of these compounds assuming similar site (mode) of nonspecific action. With this study it is shown that not only do these compounds act additively, specifically, and at the same site of action, but that regression, as suggested by Voyer and Heltshe (1984), provides an accurate prediction of the additive effect of multiple compounds acting at the same site (Figures 9-10).

Increased efforts in establishing the effects of multiple contaminants to humans and the environment is needed if future efforts to deal with environmental contamination are to be successful. In vivo and in vitro, methods of testing need to be developed and used to complement each others findings.

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VITA[2]

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